National Library of Canada
Acquisitions and Bibliographic Services Branch
395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada
Direction des acquisitions et des services bibliographiques
395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Canada

UNIVERSITY OF ALBERTA

SEROTONERGIC AND NORADRENERGIC EFFECTS ON RESPIRATORY NEURAL DISCHARGE IN THE MEDULLARY SLICE PREPARATION OF NEONATAL RATS

BY

ZAIDOON A. AL-ZUBAIDY

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

DEPARTMENT OF PHYSIOLOGY

Edmonton, Alberta

Spring 1996

National Library of Canada
Acquisitions and Bibliographic Services Branch
395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada
Direction des acquisitions et des services bibliographiques
395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-612-10691-8

Canada

UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR: ZAIDOON A. AL-ZUBAIDY

TITLE OF THESIS: Serotonergic and Noradrenergic Effects on Respiratory Neural Discharge in the Medullary Slice Preparation of Neonatal Rats.

DEGREE: Master of Science

YEAR THIS DEGREE GRANTED: 1996

Permission is hereby given to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly, of scientific purposes only.

The author reserves all other publications and other rights in association with the copyright in the thesis, and except as hereinbefore provided neither the thesis nor any portion thereof may be printed or otherwise reproduced in any material form whatever without the author's prior written permission.

30 MARCH, 96

126 Michener Park
Edmonton, Alberta
T6H 4M4

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommended to the faculty of Graduate Studies and Research for acceptance, a thesis entitled **serotonergic and noradrenergic effects on respiratory neural discharge in the medullary slice preparation of neonatal rats** submitted by **Zaidoon A. Al-Zubaidy** in partial fulfilment of the requirements for the degree of **Master of Science**.

Dr. John Greer

Dr. Christina Benishin

Dr. Jack Jhamandas

Dr. Andrew Greenshaw

Dr. Steve Harvey

Oct. 30. 1995

UNIVERSITY OF ALBERTA

SEROTONERGIC AND NORADRENERGIC EFFECTS ON DISCHARGE IN THE MEDULLARY SLICE PREPARATION

Z A. AL-ZUBAIDY, B. Erickson, and J. Greer (1996)

Permission is hereby given to the senior author (Zaidoon Al-Zubaidy) to jointly publish the above titled paper , which is the main body of his M.Sc thesis , in the " Pflugen Archiv."

Dr John Greer **R B. Erickson**

DEPARTMENT OF PHYSIOLOGY

December 20, 1995

ABSTRACT

Rhythmically active medullary slice preparations isolated from neonatal rats (P0-P3) were used to study the modulation of respiratory rhythmogenesis and hypoglossal (XII) nerve discharge by serotonin (5-HT) and noradrenaline (NA). 5-HT, NA and their respective receptor agonists and antagonists were applied either to the bathing medium or focally via pressure injection into regions encompassing the pre-Bötzinger complex (pre-BötzC) or XII motoneurons. The effects of endogenously released 5-HT were also studied by chemical stimulation of neurons within the nucleus raphé obscurus.

The frequency of respiratory burst discharge was increased when 5-HT was applied: i) to the bathing medium ($37\pm16\%$; $30\mu M$; $p<0.05$), ii) via pressure injection into the region of the pre-BötzC ($22\pm14\%$; $p<25$ pmoles; $p<0.05$) or, iii) endogenously released in response to activation of neurons within the nucleus raphé obscurus via pressure injection of AMPA ($34\pm15\%$; $p<0.05$) or 5-HT ($33\pm5\%$; $p<0.05$). All of these effects were antagonized by bath application of methysergide (30-40 μM).

NA caused a reduction of respiratory burst frequency when applied to the bathing medium ($60\pm15\%$; 100 μM; $p<0.05$) or when pressure injected into the region of the pre-BötzC ($22\pm11\%$; <25 pmoles; $p<0.05$). These effects were blocked by the bath application of α_2 adrenergic receptor antagonist idazoxan (2 μM).

5-HT and NA both caused an augmentation of tonic discharge of XII nerves when applied either to the bathing medium or via pressure injection into the XII motoneuron

pool. The 5-HT induced XII nerve tonic discharge was mimicked by the 5-HT_2 receptor agonist DOI.HCl (5 μM) and blocked by the 5-HT_2 receptor antagonist ketanserin tartrate (30-40 μM). The NA induced tonic XII nerve discharge was mimicked by the α_1 adrenergic receptor agonist phenylephrine HCl (500 nM) and blocked by the α_1 adrenergic receptor antagonist prazosin HCl (1μM).

Finally, immunocytochemistry was used to demonstrate the presence of 5-HT and catecholamine (TH) containing nerve fibres within the proposed rhythm generating centre (pre-BötzC) and the XII motoneuron pool.

ACKNOWLEDGEMENTS

I would firstly like to express my deep appreciation to my Supervisor, Dr. John Greer, for his scientific guidance and patience throughout this research. This body of work would not have been satisfactorily completed if it was not for his constructive advice and continuous professional approach. My gratitude also extends to Dr. Theodor Petrov for his supervision during my immunoctyochemical training. I would also like to thank my father, Dr. Ali J. Al-Zubaidy and Mr. Michael Wride for their help and advice during the preparation of this manuscript. A special thanks goes to Mr. Nadir Hirji for his constant friendship and support during my stay in Edmonton.

Lastly, my strongest appreciation extends to my parents and family for their constant support and encouragement.

I dedicate this thesis to my son Ali Jr.and my wife Houda.

TABLE OF CONTENTS

PAGE

List of abbreviations

Abstract

List of figures

CHAPTER 1. INTRODUCTION

1.1 Neural control of respiration 1

1.1.1 Pre-Bötzinger complex (Pre-BötzC) 1

1.1.2 Neurochemical control of respiration 5

1.2 Purpose and rationale of the study 8

1.3 Monoaminergic modulation of respiration 10

1.3.1 Effects of 5-HT on respiration *in vivo* 11

1.3.1.1 Exogenous application of 5-HT 11

1.3.1.2 Inhibiting 5-HT breakdown 12

1.3.1.3 Inhibiting 5-HT re-uptake 13

1.3.1.4 Stimulation of endogenous 5-HT release from the raphé nucleus 13

1.3.1.5 Attenuating endogenous 5-HT levels 14

1.3.2 Effects of 5-HT on respiration *in vitro* 15

1.3.2.1 5-HT applied to bathing medium 15

1.3.2.2 5-HT administered to specific medullary regions 16

1.3.2.3 Activating raphé (5-HT cells) activity 16

1.3.3 Summary of 5-HT effects; *in vivo* and *in vitro* studies 17

1.3.4 Noradrenaline (NA) 17

1.3.4.1 Effects of NA on respiration *in vivo* 18

1.3.5 Effects of NA on respiration *in vitro* 18

1.3.5.1 Decreased frequency of respiratory rhythm 19

1.3.5.2 Augmented motoneuronal activity-tonic activity 20

PAGE

1.3.6 Summary of NA effects; *in vivo* and *in vitro* studies 20

1.4 Problems associated with studying the neurochemical control of respiration 21

1.5 *In vitro* preparations used to study the neural control of respiration 22

1.6 Recapitulation of the thesis project outline 27

CHAPTER 2. MATERIALS AND METHODS

2.1 Medullary slice preparation 28

2.2 Drug application 29

2.2.1 Bath application of drugs 29

2.2.2 Pressure ejection of drugs 29

2.2.3 Drugs applied 31

2.3 Recording and analysis 31

2.4 Immunolabelling 32

CHAPTER 3. RESULTS

3.1 Effects of 5-HT on respiratory rhythm and XII nerve discharge 34

3.2 Effects of NA on respiratory rhythm and XII nerve discharge 36

3.3 5-hydroxytryptamine (5-HT) immunofluorescence 43

3.4 Tyrosine hydroxylase (TH) immunofluorescence 43

CHAPTER 4. DISCUSSION

4.1 5-HT mediated increases in respiratory frequency 46

4.2 5-HT mediated increases in tonic XII nerve discharge 48

4.3 NA mediated decreases in respiratory frequency and increases in tonic XII nerve discharge 49

PAGE

4.4 5-HT and NA containing fibres within the pre-BötzC and the XII motoneuron pool 50

4.4.1 5-HT immunofluorescence 50

4.4.2 Tyrosine hydroxylase immunofluorescence 51

4.5 Implications of state-dependant changes and the balance of monoamine levels 52

4.6 Suggestions for future work 53

REFERENCES 55

LIST OF FIGURES

Figure		Page
I-1	Schematic diagrams illustrating the location of the pre-BötzC.	6
I-2	Schematic diagrams illustrating arrangement of recording electrodes and spatial distribution of respiratory motoneuronal activity.	23
II-1	5-HT increases respiratory discharge frequency in the medullary slice preparation.	37
II-2	Activation of neurons within the nucleus raphé obscurus (NRO) via focal pressure injection of excitatory amino acid AMPA.	38
II-3	5-HT receptor mediated increase of XII nerve tonic discharge in the medullary slice preparation.	39
II-4	Location of pressure injection sites in the pre-BötzC, XII motor nucleus and nucleus raphé obscurus.	40
II-5	NA receptor mediated decreases in respiratory discharge frequency and induction of XII nerve tonic discharge in the medullary slice preparation.	42
II-6	A series of photomicrographs demonstrating the presence of 5-HT immunopositive fibres within specific medullary regions.	44
II-7	A series of photomicrographs demonstrating the presence of tyrosine hydroxylase immunopositive cell bodies and fibres within specific medullary regions.	45

LIST OF ABBREVIATIONS

Medullary regions

5 SP, spinal trigeminal nucleus.

7, facial nucleus.

DRG, dorsal respiratory group.

cNA, caudal (semicompact) division of the nucleus ambiguus.

HMN, hypoglossal motoneuron nuclei.

IV, fourth ventricle.

KF, kölliker fuse nucleus (PB/KF comprise the pontine respiratory group).

LRN, lateral reticular nucleus.

NAm, nucleus ambiguus.

NRO, nucleus raphé obscurus.

NTS, region of the nucleus solitary tract.

PB, parabrachial nuclei.

RFN, retrofacial nucleus.

RLN, recurrent laryngeal nerve.

Pre-BötzC, pre-Bötzinger complex.

PRG, pontine respiratory group.

RTN, retrotrapezoid nucleus.

SO, superior olive.

rVRG, rostral ventral respiratory group.

XII, hypoglossal nerve.

Drugs

5-HT, 5-hydroxytryptamine.

5-HTP, L-5-hydroxytryptophan

5-7-DHT, 5,7,dihydroxytryptamine.

8-OH-DPAT.HBr, R(+)-8-hydroxydipropylaminotetralin hydrobromide.

AP4, (±)-2-Amino-4-phosphonobutyric acid.

AMPA, (R,S)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid hydrobromide.

CNQX, 6-Cyano-7-nitroquinoxaline-2,3-dione.

DOI.HCl, R(-)2-(2,5-dimethoxy-4-iodophenyl) aminoethane hydrochloride.

DCBAG, 2,6,dichlorobenzylideneaminoguanidine acetate.

GABA, γ-aminobutyric acid.

m-CPBG, 1-(m-chlorophenyl)-biguanide HCl.

MK486,1-α-(3,4-dihydroxybenzyl) α-hydrazinopropionic acid.

MK801, (5R, 10S)-(+)-5 Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine.

NA, noradrenaline.

SP, substance P.

PBS, phosphate buffer solution.

pCPA, parachlorophenylalanine.

pCPEA, parachlorophenylethylalanine.

TH, tyrosine hydroxylase.

TRH, thyrotropin releasing hormone

Terminology

O.S.A.S., obstructive sleep apnea syndrome.

P.S., paradoxical sleep.

S.I.D.S., sudden infant death syndrome.

S.W.S., slow wave sleep.

Routes of administration

i.c.v., intracerebroventricular.

i.p., intraperitoneal.

i.v., intravenous.

p.o., oral.

CHAPTER 1

1. INTRODUCTION

1.1 *Neural control of mammalian respiration*

The neural control of mammalian respiration has been of interest to neurobiologists for centuries. Particular attention has focused on the identification of the site responsible for respiratory rhythmogenesis and the neurochemical factors that control neuronal activity within this location. Early studies involved rather crude lesioning of various regions within the pons, medulla and spinal cord of anesthetized animals. Not surprisingly, many of these lesions resulted in some type of perturbation of respiratory motor discharge, however no specific nucleus could be clearly identified as being the source of rhythmogenesis (Feldman, 1986). Recently, finer lesioning techniques using an *in vitro* preparation derived from neonatal rats (described below), have led to the identification of a well-defined region within the ventrolateral medulla which is currently thought to be the primary sight for respiratory rhythmogenesis (Smith et al. 1991). This area is referred to as the pre-Bötzinger complex (pre-BötzC).

1.1.1 *Pre-Bötzinger complex (pre-BötzC) - putative medullary region responsible for respiratory rhythm*

There are three concentrated regions of respiratory-related neurons within the brainstem; the pontine respiratory group (PRG), dorsal medullary respiratory group

(DRG) and the ventral medullary respiratory group (VRG). For the purposes of discussing the site of respiratory rhythmogenesis, we need only be concerned with the VRG. The VRG consists of a column of cells within the ventrolateral medulla in the region of the nucleus ambiguus and nucleus retroambigualis, spanning from the spinomedullary border rostral to the retrofacial nucleus (Fig. I-1). This column of cells is not homogeneous with regard to the functional properties of respiratory neurons. Populations of predominantly expiratory neurons are found in regions of the VRG caudal to the obex (region near the floor of the fourth ventricle). The intermediate regions of the VRG contain a large proportion of inspiratory neurons. Another distinct population of expiratory neurons are found rostral to the rVRG and are located medial to the retrofacial nucleus. This group of expiratory neurons is referred to as the Bötzinger complex (the name Bötzinger was rather arbitrarily adopted at a respiratory control meeting held in 1980 adjacent to the Bötzinger vineyard near Heidelberg). An area which is thought to contain the neuronal substrate underlying respiratory rhythmogenesis is situated between the rostral portion of the ventral respiratory group (rVRG) and the Bötzinger complex (Smith et al. 1991) , and it has been referred to as the pre-BötzC (Fig. I-1). It has been clearly demonstrated that this discrete region of the medulla, when maintained *in vitro*, is capable of generating a respiratory related rhythm (Smith et al. 1991) . There is currently no evidence to show that any other region of the neuraxis can generate respiratory related oscillations. Further studies, utilizing neonatal rat *in vitro* and *in vivo* rat and cat models, have demonstrated that pharmacological blockade of neuronal activity or lesioning of this region of the brainstem results in the elimination of respiratory related neural discharges (Smith et al.

1991, Connelly et al. 1991; Funk et al, 1993). Thus, the current working hypothesis is that the pre-Bötzinger region contains the kernel of cells accountable for respiratory rhythmogenesis (Smith et al. 1991).

The pre-BötzC contains a population of neurons which have characteristics typical of conditional pacemaker neurons. These cells generate large membrane potential oscillations and bursts of action potentials when depolarized into the -55 mV range. The time interval between these oscillations decreases as the membrane is further depolarized. In contrast, neurons in adjacent regions respond differently by simply displaying a series of action potentials in response to a depolarizing current (Smith et al. 1991). The pre-BötzC is not exclusively comprised of such pacemaker-like cells, however. Neurons displaying inspiratory-modulated, expiratory-modulated and phase spanning activity have also been found within this region (Smith et al. 1991; Connelly et al. 1992a; Schwarzacher et al. 1995).

Histological evidence utilizing retrograde tracing techniques has shown that the pre-BötzC also has anatomical features distinct from the rostral and caudal regions of the VRG. Regions of the VRG outside of the pre-BötzC contain large populations of bulbospinal neurons and cranial motoneurons (IX and X). In contrast, within the pre-BötzC, there is a relative paucity of these cell types and a high density of propriobulbar neurons (Smith et al 1991; Dobbins and Feldman 1994). The presence of a high concentration of interneurons is in agreement with the idea of the pre-BötzC being a central source for the transmission of respiratory rhythm to other respiratory-related regions including premotoneurons and descending bulbospinal premotoneurons (Smith et

al. 1991).

Currently, the pre-BötzC region is being investigated both *in vitro* and *in vivo* in hopes of determining the neuronal mechanisms underlying respiratory rhythm generation. There are two divergent views regarding the generation of respiratory rhythm. One view states that a population of pacemaker cells (Onimaru et al. 1992) are responsible for generating the basic respiratory rhythm. In support of this view, as stated above, neurons with pacemaker-like properties have been identified in the pre-BötzC region *in vitro*. However, no causal link between the discharge of these pacemaker-like neurons and respiratory rhythmogenesis has been demonstrated. Moreover, neurons displaying pacemaker-like properties have not been described within the pre-BötzC of any preparation other than the medullary slice.

The other view of rhythmogenesis proposes that the rhythm arises as a result of a network of interconnected neurons, none of which are endowed with the ability to oscillate on their own (Richter 1982). A number of network models which incorporate the drive potential profiles of various classes of inspiratory, expiratory and phase-spanning neurons has been generated. However, there are two fundamental problems with these models. First, not all of the respiratory neuron types so far recorded from (particularly those located within the pre-BötzC) can be incorporated into the models. Second, chronic *in vivo* recordings have demonstrated that a given neuron's firing pattern can change from one pattern to another depending on the behavioral state of the animal (Chang 1993; Orem 1994). Thus, any model would have to account for such pliable neuronal firing patterns. A hybrid of the two views has been recently suggested which proposes that there

is a population of conditional pacemaker neurons (i.e. neurons which oscillate when provided with the appropriate depolarizing drive) which are influenced and controlled by a network of cells in which they are imbedded (Smith et al. 1992). In summary, while the region of the medulla responsible for generating respiratory oscillations has been determined, the underlying neuronal mechanisms responsible for generating the rhythm remain unclear. However, now that there is a well-defined target within the medulla it is likely that future recordings from populations of neurons within the pre-BötzC will provide some more definitive data towards solving this fundamental and long-standing problem.

1.1.2 *Neurochemical control of respiration*

Excitatory amino acids (EAA), acting predominantly on non-NMDA receptors, play an important role in both respiratory rhythmogenesis and the transmission of inspiratory drive to the spinal and cranial inspiratory motoneurons in the mammalian nervous system. *In vitro* studies utilizing the isolated neonatal rat brainstem-spinal cord and medullary slice preparations, have shown that the frequency of spontaneous respiratory bursting is decreased in a dose-dependent manner by the addition of the non-NMDA receptor antagonist CNQX (Greer et al. 1991; Funk et al. 1993). Furthermore, when the levels of endogenously released EAAs are amplified, by the addition of EAA uptake inhibitors to the bathing medium, there is a dose-dependent increase in respiratory discharge frequency. These results suggest that EAAs acting at non-NMDA receptors (AMPA and/or kainate) are providing a conditioning drive to maintain

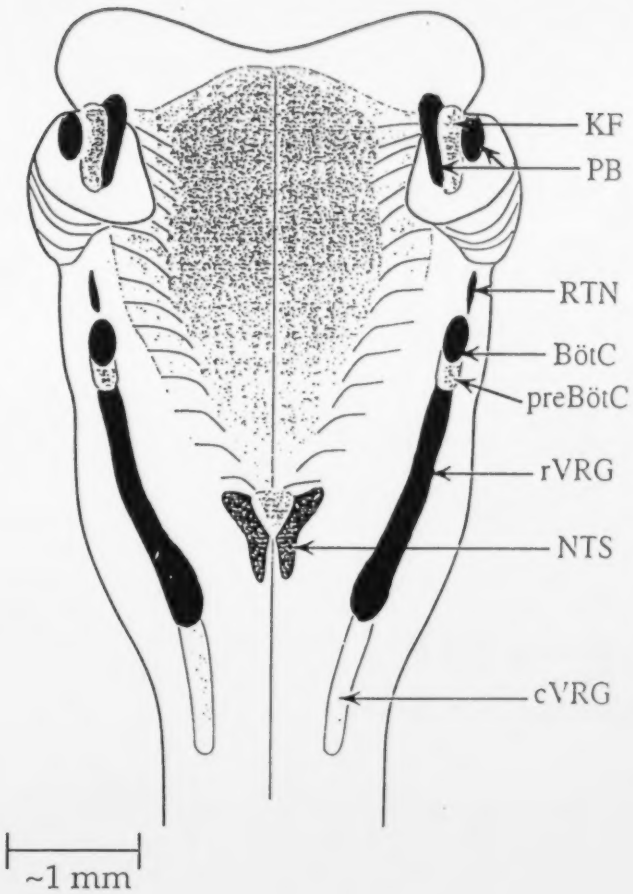
Fig. I-1

A) Dorsal view of the brainstem illustrating the location of the pre-Bötzinger complex (pre-BötzC), in relation to the other brainstem regions involved in the control of breathing.

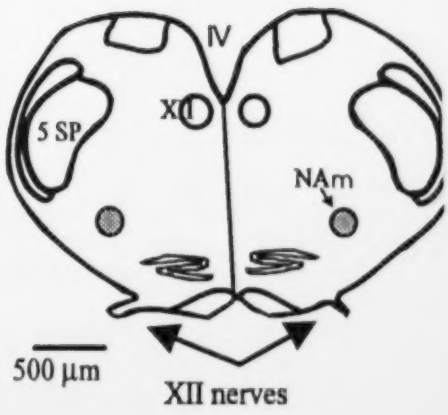
B) A schematic diagram illustrating a transverse section of the medullary slice preparation.

Fig. 1A is adopted from Feldman, J.L. (1986). Neurophysiology of breathing in mammals. Handbook of Physiology, section 1: The Nervous System, vol. 4, Intrinsic Regulatory Systems of the Brain. (FE Bloom, Ed.). Bethesda. MD, American Physiology Society. 463-524.

A


KF
PB
RTN
BötC
preBötC
rVRG
NTS
cVRG
~1 mm

B


IV
XII
5 SP
NAm
500 µm
XII nerves

and modulate rhythmogenesis. Blockade of EAA receptors within the pre-BötzC *in vivo*, likewise, leads to perturbations of respiratory frequency (Connelly et al. 1992b).

In vitro preparations have also been utilized to investigate the roles of pre-BötzC in the transmission of inspiratory drive to cranial and spinal motoneurons. The amplitude of inspiratory population discharge of cranial (IX, X, XII) and spinal (C1,C2,C4 and thoracic) motoneurons is decreased in a dose dependent manner by CNQX (Lui et al. 1990; Greer et al. 1991; Funk et al. 1993) and increased by EAA uptake inhibitors (McCrimmon et al. 1989) when applied to the brainstem spinal cord preparation's bathing medium. Glutamate-mediated inspiratory drive transmission to phrenic motoneurons is also depressed by the presynaptic actions of AP4-sensitive receptors (Greer et al. 1992a).

While EAA seem to be responsible for maintaining the basic respiratory rhythm and drive transmission, a number of other neuromodulators have been implicated in the control of breathing. Neurotransmitters such as monoamines (Lalley, 1986; Hilaire et al. 1989), acetylcholine (Champagnat et al. 1979; Murakoshi et al. 1985), endogenous opioids (Greer et al. 1995b; Moss and Inman 1989) and thyrotropin releasing hormone (TRH) (Greer et al. 1995a), either augment or attenuate respiratory frequency. Particular attention has been focussed on the two monoamine neurotransmitter systems addressed in this thesis, serotonergic (5-HT containing) and noradrenergic, due to their diverse roles in various behavioral functions that affect respiration, such as sleep (Jouvet 1969).

The role of neuromodulators in controlling inspiratory drive transmission to cranial and thoracoabdominal motoneuronal pools is also receiving considerable attention. There is evidence that postsynaptic receptors for GABA, serotonin (5-HT), noradrenaline (NA),

substance P, neuropeptide Y, galanin, metenkephalin, TRH are present in the phrenic nucleus (Ellenberger et al. 1992). Presynaptic 5-HT (Lindsay and Feldman 1993), $GABA_B$, glutamate (via AP4) receptors have also been identified (Lui et al. 1990). A relevant question is, why are there so many neurotransmitters required to modulate respiratory drive in addition to glutamate (excitatory) (Lui et al. 1990) and GABA (inhibitory) (Fedorko et al. 1987)? It may reflect the fact that different neurotransmitter systems modulate various behaviours, which in turn place demands on respiratory function, for example, to increase/decrease frequency of breaths, or the tidal volume. Indeed, it has been proposed that the diversity of neurotransmitter receptors in the phrenic nucleus ensures that breathing is adjusted to the various respiratory demands that different behaviours may exert (Feldman, 1986).

1.2 *Purpose and rationale of the study*

The purpose of this study was to provide further information concerning the neurochemical control of respiration. This study specifically focussed on serotonergic and noradrenergic influences on respiratory frequency and the inspiratory drive to motoneurons (XII) controlling upper airway patency. The experimental model utilized in these studies was the medullary slice *in vitro* preparation isolated from neonatal rats. This preparation contains the respiratory rhythm generating centre (pre-BötzC) and a population of XII motoneurons. Moreover, the preparation spontaneously generates a respiratory-related motor discharge *in vitro* and is thus suitable for pharmacological studies which assay the effects of particular neurotransmitter receptor agonists and

antagonists. The following four fundamental questions were addressed:

1. Do 5-HT and NA modulate both the frequency of respiratory rhythm and the amplitude of hypoglossal motoneuronal discharge?
2. Which regions of the brainstem mediate the 5-HT and NA induced effects on respiratory rhythm and inspiratory drive transmission to hypoglossal motoneurons?
3. Which receptor subtypes are responsible for the differing effects of 5-HT and NA on respiration?
4. Are there monoaminergic projections innervating the pre-BötzC and XII motoneuron pool?

Four experimental approaches were used to address these questions. 1) Agonists and antagonists to 5-HT and NA receptors were added to the medium bathing medullary slice preparations. ii) Agonists and antagonists were applied focally, via pressure ejection, to regions of the pre-BötzC and XII motoneuron pool. iii) The effects of endogenous 5-HT release, induced by pressure ejection of AMPA and 5-HT into the nucleus raphé obscurus (which is also contained within the medullary slice) were assayed. iv) Finally, the presence of 5-HT and catecholamine containing fibres within the pre-BötzC and XII motoneuron pool were determined with immunohistochemical labelling.

Before proceeding with the presentation of this research, I will specifically discuss previous work related to the role of 5-HT and NA in the control of breathing and further discuss the medullary slice preparation.

1.3 *Monoaminergic modulation of respiration*

There is a particular interest in the role of serotonergic and noradrenergic transmitter systems in the modulation of breathing during different sleep states. The activity of ponto-medullary noradrenergic and serotonergic neurons fluctuate during waking and various sleep states (Jones, 1991). Moreover, previous studies have shown that these two neurotransmitters have profound effects on both breathing frequency and the amplitude of respiratory motoneuron activity (Champagnat et al. 1979; Lalley, 1986; Millhorn, 1986; Hilaire et al. 1989; Monteau et al. 1990; Berger et al. 1992; DiPasquale et al. 1992; Kubin et al. 1992; Morin, 1993; Funk et al. 1994). Therefore, elucidating the underlying neuronal mechanisms of serotonergic and noradrenergic actions within respiratory rhythm generating centres and medullary motoneuron pools, potentially has significance for the diagnosis and therapy of perinatal breathing disorders as well as for the design and administration of monoaminergic drugs alleviating respiratory distresses such as obstructive sleep apnea syndrome (OSAS).

The presence of extensive axonal connections from medullary 5-HT containing raphé nuclei (nucleus raphé magnus and the nucleus raphé obscurus) and the catecholaminergic cells (medullary C1 and C2) to the ventrolateral medulla, suggests that these monoaminergic nuclei have a role in affecting respiratory drive (Dahlström and Fuxe 1964; Levit and Moore 1979; Aldes et al. 1988; Aldes et al. 1989; Holtman 1990; Pilowski et al. 1990, 1994; Sun et al 1994). Numerous pharmacological investigations have shown that influencing brainstem monoaminergic activity effects respiration. Such

studies have ranged from neurotoxic lesioning of monoaminergic medullary cells to the administration of monoaminergic receptor agonists to respiratory neurons (Eldridge and Millhorn 1981). However, as outlined below, many of the pharmacological studies have generated contradictory data.

1.3.1 *Effects of 5-HT on respiration in vivo*

Numerous investigations *in vivo* have focussed on modulating 5-HT levels in various animal models, in order to study the effects of 5-HT on respiration centrally. Increasing 5-HT's synaptic levels can be achieved by either directly injecting 5-HT into the model of interest, inhibiting the uptake mechanism, inhibiting enzymic breakdown or stimulating raphé (5-HT containing cells) activity. Endogenous 5-HT levels have been decreased by administering drugs which block 5-HT synthesis and chemical lesioning of 5-HT neurons. Finally, receptor antagonists have been administered to block the actions of endogenously released 5-HT.

1.3.1.1 *Exogenous application of 5-HT*

The effects of administering 5-HT systemically are to be ignored here because 5-HT is unable to cross the blood-brain-barrier, therefore any result obtained is peripheral in origin. However, systemic administration of L-5-hydroxytryptophan (5-HTP), a 5-HT precursor which crosses the blood-brain-barrier, produces an increase in the frequency of respiratory rate in various species (Bogdanski et al. 1958). There is also evidence showing that i.v. administration of 5-HTP causes inhibition of the amplitude of phrenic

nerve activity in cats (McCrimmon and Lalley 1982).

Injections of 5-HT i.c.v., causes a facilitatory effect on the respiratory rate (number of breaths per minute) in oxen (Findlay and Thompson 1968) and in awake (Gaddum and Vogt 1956) and anaesthetized cats (Felberg and Sherwood 1954). However, in another study in cats, 5-HT induced a biphasic effect, i.e. facilitation followed by a reduction in respiratory frequency (Lambert et al. 1978).

Studies involving 5-HT injections directly into the brainstem respiratory nuclei have also generated contradictory results. 5-HT iontophoretically applied to what are described as "spontaneously active brainstem neurons" confined to the ventral surface of the medulla, rostral to the level of the obex, caused prolonged excitation of neuronal discharge (Boakes et al. 1970; Bramwell and Bradley 1974). In other studies, 5-HT focally applied to inspiratory neurons in the dorsal and ventral surfaces of the same region, caused excitation in half of the inspiratory neurons tested and inhibition in the other half (Hölsi et al. 1971; Champagnat et al. 1979; Arita and Ochiishi 1991). Arita and Oochiishi proposed that the inconsistent findings were due to the presence of two subpopulations of inspiratory neurons. 5-HT induced an excitatory response in the majority of decrementing inspiratory neurons (83%), whereas, incrementing inspiratory neurons were mostly inhibited (77%).

1.3.1.2 *Inhibition of 5-HT breakdown*

5-HT is broken down within the synapse by the enzymes monoamine oxidase$_A$ and monoamine oxidase$_B$ (MAO_A and MAO_B). Therefore, inhibiting these enzymes results in

an elevated synaptic concentration of 5-HT. In vagotomized, paralysed, chemodenervated cats and freely moving rats, injections of MAO inhibitors (i.c.v. and i.p.) led to prolonged increases in the amplitude of phrenic nerve discharge and respiratory frequency (Sloviter et al. 1978; Eldridge et al. 1979).

1.3.1.3 *Inhibition of 5-HT re-uptake*

5-HT and other biogenic amines are taken-up into their respective presynaptic vesicles as a mechanism of regulating synaptic neurotransmitter levels. Blocking this re-uptake mechanism results in increased synaptic neurotransmitter levels. The administration of monoamine uptake blockers in cats, has been reported to cause an augmentation of hypoglossal (XII) and recurrent laryngeal nerve (RLN) activity (Bonora et al. 1985).

1.3.1.4 *Stimulation of endogenous 5-HT release from the raphé nucleus*

The activation of 5-HT containing cell bodies (raphé nuclei), by electrical or chemical stimulation, is a method by which 5-HT can be released from an endogenous source. Electrical stimulation of the nucleus raphé obscurus in the anesthetized cat results in an increase in the amplitude and respiratory frequency of neural discharges recorded from the phrenic nerve. This effect was reversed upon application of a 5-HT antagonist, methysergide (i.v.), indicating that the effect observed was mediated by 5-HT (Millhorn 1986). However, a similar experiment, conducted under the same conditions produced a depression of respiratory amplitude and frequency (Lalley, 1986). It should be noted, however, that experiments utilizing electrical stimulation can produce misleading results.

For example, 5-HT coexists with substance P (SP) and TRH within the raphé nuclei (Törk, 1985). These three neuromodulators are either released differentially or together depending on the strength of the electrical stimulation. Therefore, when interpreting data generated from electrical stimulation of 5-HT containing cells, one must consider that the results can be caused by combinations of any of the three coexisting neuromodulators. Lateral spread of the stimulus to adjacent regions is another factor to consider when interpreting electrical stimulation data. Such a spread may influence the activity of an adjacent region which may not be normally recruited. Chemical activation of the nucleus raphé obscurus by pressure injection of 5-HT or glutamate *in vivo* causes a 5-HT mediated excitation of neuronal activity within the nucleus raphé obscurus (Dreteler et al. 1991; Holtman et al. 1986, 1987) which in turn results in an increased amplitude of integrated phrenic nerve activity as well as an augmentation of respiratory frequency. Intraperitoneal (i.p.) injections of compounds inducing the release of 5-HT, such as parachlorophenylethylalanine (pCPEA), have been shown to increase the respiratory frequency in freely moving rats (Sloviter et al. 1978).

1.3.1.5 *Attenuation of endogenous 5-HT levels*

Inhibiting 5-HT synthesis by systemic administration of agents such as parachlorophenylalanine (pCPA) (tryptophan hydroxylase inhibitor), in awake rats, results in an augmentation of respiratory frequency (Olson et al. 1979). Based on this pharmacological finding, it was proposed that 5-HT inhibits the central nervous system output, which controls normal breathing. Chemical lesioning of 5-HT neurons, by i.c.v.

injection of the 5-HT neurotoxin 5,7,dihydroxytryptamine (5-7-DHT) caused a persistent depression of respiratory frequency (Olson et al. 1979). Inhibiting 5-HT postsynaptic actions by i.v. administration of the 5-HT antagonist methysergide produced an immediate depression of respiratory frequency in cats (Millhorn et al. 1980). Thus, these latter mentioned results suggest that 5-HT is a facilitatory modulator of respiration.

1.3.2 *Effects of 5-HT on respiration in vitro*

In order to study 5-HT's modulatory effects on respiration *in vitro*, different preparations have been utilized, such as thin medullary slices and the isolated neonatal rat brainstem-spinal cord preparation. 5-HT's effects on respiratory neuronal activity were investigated by either applying 5-HT to the preparation's bathing medium, pressure injection of 5-HT into various medullary regions, or by inducing endogenous release of 5-HT via stimulating the nucleus raphé obscurus. However, such investigations have also yielded inconsistent data.

1.3.2.1 *5-HT applied to bathing medium*

The application of 5-HT to the bathing medium of the neonatal isolated brainstem spinal cord preparation caused an increase in the frequency of rhythmic bursting and an excitation of phrenic motoneuron discharge (Murakoshi et al. 1985; Monteau et al. 1990; Morin et al. 1990a,b, Morin 1993; Di Pasquale et al. 1992; Lindsay and Feldman 1993). The results of 5-HT application to cranial motoneurons were equivocal. Morin et al. reported a strong inhibition of the amplitude of hypoglossal (XII) nerve discharge.

However, intracellular recordings from XII motoneurons contained within 130μm or 400-500μm thick slice preparations derived from juvenile and adult rats clearly showed that 5-HT depolarized XII motoneurons (Berger et al. 1992). It should be noted that these slices do not generate respiratory rhythms and thus it is not possible to differentiate respiratory from non-respiratory XII motoneurons.

1.3.2.2 *5-HT administered to specific medullary regions*

5-HT injected into the ventrolateral medulla of the isolated neonatal brainstem spinal cord preparation elicited an increase in the frequency of respiratory bursts (DiPasquale et al. 1992). Unfortunately, these investigators did not determine the location of the injection sites other than indicating that they were within the vicinity of the rostral ventrolateral medulla.

1.3.2.3 *Activating raphé (5-HT cells) activity*

The stimulation of medullary raphé nuclei (nucleus raphé obscurus) chemically (via pressure injection of glutamate) or electrically caused an increase in the frequency of respiratory bursts and a depression of the amplitude of motoneuronal inspiratory discharge in the spinal (C4) and hypoglossal (XII) nerves (Morin et al. 1990b). These findings suggest that 5-HT's effects originate from the raphé nuclei to stimulate the respiratory centre responsible for governing respiratory rhythm and depress cranial and spinal motoneuronal populations.

1.3.3 *Summary*

5-HT's role in modulating respiratory activity still remains unclear, despite the different approaches used to study its effects. Both *in vivo* and *in vitro* studies have shown that 5-HT induces either excitatory, inhibitory or biphasic effects on respiratory rhythm. Investigating the effect of 5-HT on motoneuronal activity has also generated inconsistent findings. Such equivocal results may be due to the difficulties associated with the route of administration of 5-HT and the limitations of the models used. The merits and drawbacks associated with using different experimental models to study the neurochemical control of respiration will be comprehensively discussed later in this chapter.

1.3.4 *Noradrenaline (NA)*

The locus coerulus (LC) and other NA containing group (such as the locus subcoerulus, C1, C2, A5) have diffuse axonal pathways. Immunohistochemical studies have demonstrated the presence of NA containing terminals within populations of medullary respiratory related neurons (Sun et al. 1994). It is therefore assumed that NA plays a modulatory role in mediating respiration. There appears to be a general agreement with respect to certain aspects of the central effects of NA on respiration. Several investigators, utilizing both *in vivo* and *in vitro* models, have reported that NA has a depressant effect on respiratory rhythm. The actions of NA on XII motoneuron respiratory discharge is less clear, as some studies report an excitation, while others report no effect (Bolme and Fuxe 1973; Champagnat et al. 1979; Murakoshi et al. 1985, Hilaire et al. 1989; Errichidi et al. 1990, 1991; Arita and Oochiishi 1991; Parkis et al.

1994; Funk et al. 1994; Zou 1994).

1.3.4.1 *Effects of NA on respiration in vivo*

Peripheral administration of adrenergic agonists appears to exert an excitatory effect on respiration. This is attributable to the stimulation of cardiopulmonary adrenergic receptors, resulting in cardiac excitation and arterial constriction (Bowman and Rand 1980). This effect is in turn relayed to the brainstem to induce an increase in respiratory frequency to accommodate for this behaviour. Adrenaline and NA also cause excitation of peripheral chemoreceptors which evokes an increase in respiratory activity (Moss and Inman 1989).

Centrally, NA has an inhibitory effect on respiration. Administration of adrenergic receptor agonists 1-α-(3,4-dihydroxybenzyl) α-hydrazinopropionic acid (MK 486) and 2,6,dichlorobenzylidene aminoguanidine acetate (DCBAG) (i.p. and i.v.) produced a decrease of respiratory rate in rats (Bolme and Fuxe 1973). In other studies, using cat and opossum models, focal administration of NA within populations of decrementing and augmenting inspiratory neurons caused a decrease in inspiratory neuronal firing and breathing frequency (Champagnat et al 1979; Arita and Ochiishi 1991; Funk et al. 1994; Zou 1994).

1.3.5 *Effects of NA on respiration in vitro*

Various *in vitro* preparations, such as the isolated brainstem spinal cord preparation and thin medullary slices, have been utilized to examine NA's effect on

respiration (Murakoshi et al. 1985; Hilaire et al. 1989; Errichidi et al. 1990,1991; Parkis et al. 1994; Funk et al. 1994). NA, applied to the isolated neonatal rat brainstem spinal cord preparation and the medullary slice, causes a decrease in the frequency of respiratory rhythm. However, there have been contradictory reports regarding the actions of NA on XII motoneuron discharge with one group reporting no effect (Hilaire et al. 1989) and two other studies clearly showing an excitatory effect (Parkis et al. 1994; Funk et al. 1994).

1.3.5.1 *Decreased frequency of respiratory rhythm*

Electrical stimulation of the pontine noradrenergic A5 region in the isolated brainstem spinal cord preparation elicits a decrease in the frequency of respiratory rhythm This demonstrates that projections from structures located within the caudal ventrolateral pons can depress the respiratory rhythm generator. Pharmacological experiments suggest a noradrenergic origin for this inhibitory drive. Adrenergic α_2 receptor antagonists (yohimbine and idazoxan) were applied to the bathing medium to block the actions of the endogenous release of NA from the A5 group within the attached pons and a dose-dependant increase in the rate of respiratory frequency was observed. Administration of idazoxan to the medium bathing the brain stem region of the preparation (in this case with the pons removed) reversed the depression of respiratory frequency produced by exogenous application of NA, further implicating the α_2 adrenergic receptor in respiratory modulation. (Hilaire et al. 1989; Errichidi et al. 1990, 1991).

1.3.5.2 *Augmented motoneuronal activity-tonic activity*

Administering NA to the isolated brainstem spinal cord preparation resulted in an augmentation of phrenic motoneuronal activity and an increase in the amplitude of inspiratory discharges. This effect was mimicked by the α_1 adrenergic receptor agonist phenylephrine and blocked by the α_1 adrenergic receptor antagonist prazosin (Errichidi et al. 1991).

However, tonic motoneuronal depolarization was never observed from hypoglossal nerve roots of the isolated brainstem spinal cord preparation (Hilaire et al. 1989; Errichidi et al. 1990,1991). This is in contrast to the findings of Parkis et al. (1994) and Funk et al. (1994) utilizing medullary slices. NA focally applied to the hypoglossal motoneuron pool depolarized XII motoneurons in those studies.

1.3.6 *Summary*

NA appears to induce an inhibitory effect on the frequency of respiratory discharge (Champagnat et al 1979; Arita and Ochiishi 1991, Zou 1994). The receptor subtype responsible for mediating this effect is proposed to be the adrenergic α_2 receptor subtype (Errichidi et al. 1990, 1991). Excitation of cervical respiratory motoneurons induced by NA was determined to be due to the activation of the adrenergic α_1 receptor subtype encompassing the motoneuron nuclei. However, the actions of NA on XII motoneuron discharge are unclear.

1.4 *Problems associated with studying the neurochemical control of respiration*

In order to accurately determine the effects of 5-HT and NA on respiration at a central level, respiratory centres within the ventrolateral medulla should be directly affected, without affecting sources which may indirectly alter respiratory drive. In many past studies, this has not been achieved and perhaps accounts for some of the equivocal results produced. With oral (p.o.), intravenous (i.v.), intraperitoneal (i.p.) and intracerebroventricular (i.c.v.) applications, it is difficult to control the concentration and range of dispersal of a drug within the CNS. There are several indirect physiological parameters which could be altered to influence respiration, including, behavioral state, body temperature, blood gas levels and blood pressure. Another factor which may account for the discrepancies observed thus far is the presence of chemoreceptors in animal models. Chemoreceptors are responsible for detecting and monitoring blood and extracelluar fluid levels of respiratory related metabolites such as oxygen, carbon dioxide and pH. In turn, these chemoreceptors alter respiratory function in accordance to the levels detected. Without chemodenervating animals, effects observed may be due to peripheral or central chemoreceptor stimulation. Finally, there is also the added complication of determining compound specificity. For instance, suggesting that 5-HT is responsible for inducing a given effect in studies where re-uptake blockers and MAO inhibitors are used is problematic. These compounds can also affect the levels of other monoamines such as NA and dopamine. Therefore, when interpreting neuropharmacological data, all of the factors above must be taken into consideration.

1.5 *In vitro preparations used to study the neural control of respiration*

Various *in vitro* preparations have been developed to overcome some of the problems associated with the *in vivo* models. The types of *in vitro* preparations used to study the neurochemical control of respiration can be broadly categorized into those displaying spontaneous rhythmic activity and those without endogenous rhythmic activity. In experiments utilizing preparations without endogenous rhythmic activity, such as standard brain slices, it is difficult to predict whether a given cell would normally be involved in respiration in the intact brain. Moreover, in order to investigate neuronal behaviours in such a preparation, cells are often either chemically or electrically activated. Electrical stimulation may activate inputs that are not involved in respiration. Exogenous application of chemicals, to a particular region of the preparation, may also generate extraneous results.

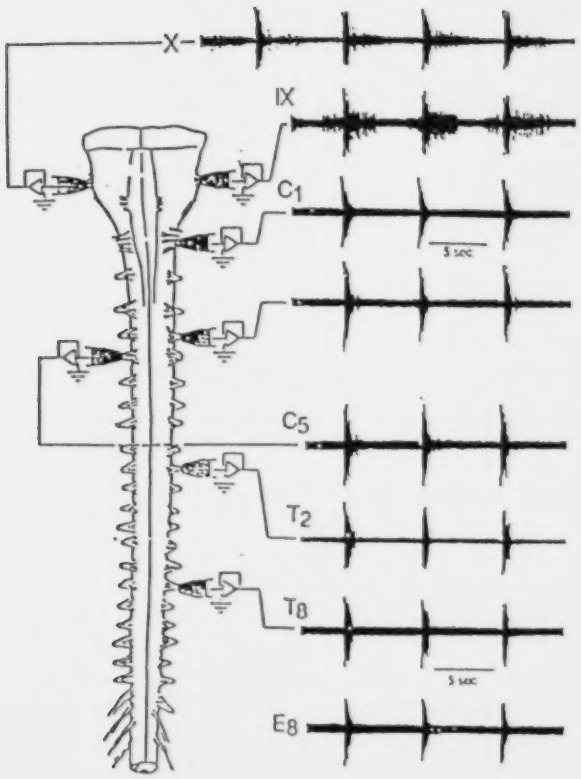
Preparations such as the isolated neonatal rat brainstem spinal cord and the medullary slice generate spontaneous respiratory-related neural discharges. The isolated neonatal (0-4 days old) brainstem spinal cord preparation (Suzue 1984) displays periodic neural activity on cranial nerves (IX, X, XII) and spinal ventral roots (cervical and thoracic) which is characteristic of respiratory motor discharge (Fig. I-2A). The medullary slice preparation (Smith et al. 1991) is a reduced version of the brainstem spinal cord preparation, consisting of 500-650 μm slice of the neonatal rat medulla (Fig. I-2B). As discussed in the beginning of the introduction, this region (pre-BötzC) contains neurons making-up the respiratory rhythm generating centre. In this slice, spontaneously generated rhythmic motor discharge can be recorded from the hypoglossal nerve.

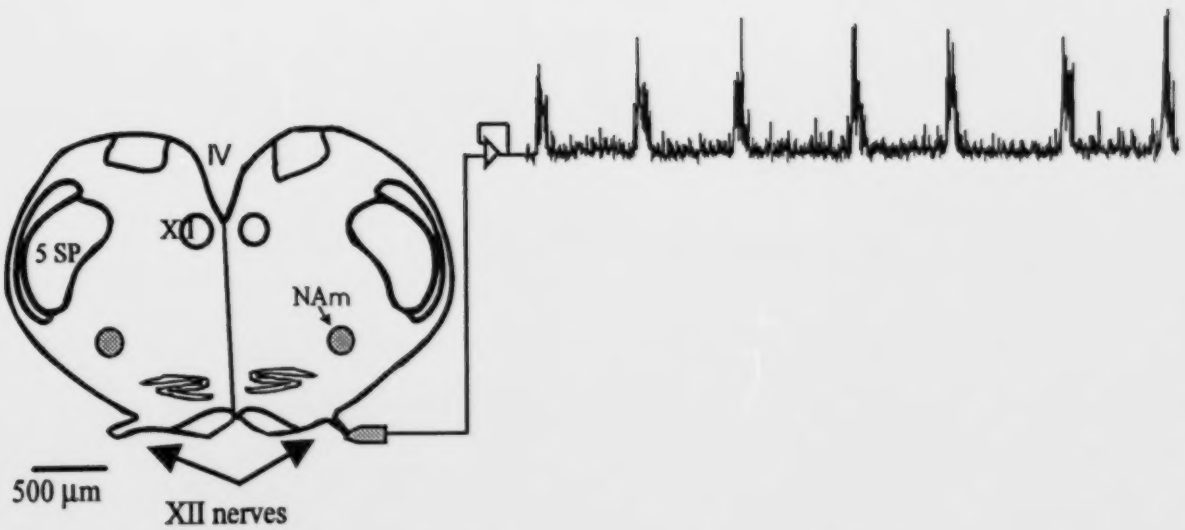
Fig. I-2 Schematic diagrams illustrating arrangement of recording electrodes and spatial distribution of respiratory motoneuronal activity.

A) A schematic diagram illustrating the distribution of spontaneous motor activity recorded from cranial [vagal (X), glossopharyngeal (IX)] and spinal motoneuron discharge (C1, C4, C5, T2 and T8) on ventral roots.

B) A schematic diagram showing a trace of respiratory motor discharges recorded from the hypoglossal nerve of a medullary slice preparation.

Fig. 2A is adopted from Smith, J.C., Greer, J.J., Lui, G. and Feldman, J.L. (1990). Neural mechanisms generating respiratory pattern in mammalian brainstem-spinal cord *in vitro*. I Spatiotemporal patterns of motor and medullary neuron activity. *Journal of Physiology* **354**:173-183.


X
IX
C1
C5
T2
T8
E8


IV
XII
5 SP
NAm
500 µm
XII nerves

Both of these spontaneously active *in vitro* preparations are well-suited for neuropharmacological studies. The relative ease by which the tissues' extracellular environment is controlled, such as the temperature, pH and ionic composition, provides added advantages of using such preparations. The lack of afferent inputs from peripheral organs, such as aortic or carotid chemoreceptors, eliminates influences that may affect respiration from these sources. Both preparations are mechanically stable, which makes them suitable for utilizing various electrophysiological and pharmacological techniques (i.e. whole cell patch clamp and focal iontophoretic administration of drugs without cardiopulmonary induced movements). Added advantages of utilizing the medullary slice preparation include the accessibility of medullary regions for direct manipulations and the lack of superfluous medullary and pontine structures that may respond to neuromodulators and indirectly interact with the pre-BötzC.

However, criticisms have arisen as to the relevance of these preparations with respect to the simulation of respiration. These preparations will only generate a spontaneous respiratory rhythm when isolated from neonatal animals (0-4 days old; P0-P4). Therefore, when interpreting neuropharmacological data, it is important to recognize that such data apply to the newborn and not necessarily to adults. The question of the relative maturity of the neuronal system has to be considered. However, it is worth noting, that in all mammals studied to date, the respiratory system is capable of generating breathing movements in utero. Fetal breathing movements are necessary for the proper development of lung, muscle and perhaps respiratory neurons. Naturally, the respiratory system must be fully operational at birth to ensure viability. Thus, there is

reason to believe that many of the basic features of the neuronal control mechanisms associated with breathing are relatively mature at birth, with perhaps the modulatory inputs changing postnatally. For example, the α_1 adrenergic receptor subtype is expressed earlier than the α_2 subtype in the neonatal rat's dorsal motor nucleus of the vagus (Fukuda et al. 1989). Our previous studies of opioid receptor actions on neonatal breathing, show an age-dependent expression of opioid receptor subtypes (Greer et al. 1995b). Also, TRH focally applied to the XII motor nucleus does not elicit an effect on XII inspiratory burst amplitude in slices from P0-P3 mice, however, by P21 a significant augmentation of motoneuronal activity is observed (Funk et al. 1994).

Another concern regarding the relevance of these *in vitro* preparations pertains to the temporal pattern of the motor output. Suzue (1984) suggested that the pattern of the motor output is similar to that of "gasping", therefore the preparation may not necessarily be breathing. This is further reinforced by criticism that the preparation's extracellular environment is hypoxic, since anoxic conditions lead to gasping in adults. However, later studies demonstrated that the partial pressure of oxygen and pH in the regions critical to respiratory function are consistent with aerobic metabolism and conventional neuronal function (Brockhaus et al. 1993). With respect to the temporal pattern of respiratory discharges, inspiratory modulated neural bursts generated *in vivo* from a vagotomized animal are similar to those of the *in vitro* preparations (Smith et al. 1990). Detailed studies of the discharge patterns of medullary and spinal respiratory neurons *in vitro* have also demonstrated the similarities between the spatiotemporal organization of respiratory patterns *in vitro* and *in vivo* (Smith et al. 1990).

While the brainstem-spinal cord preparation maintains a rhythmical motor discharge when bathed in standard artificial CSF, the medullary slice requires additional priming. Typically, medullary slices do not generate a spontaneous rhythmic motor discharge unless the potassium concentration in the bathing medium is elevated to 9 mM. It is thought that the elevated extracellular potassium depolarizes neurons responsible for generating respiratory rhythm sufficiently to activate the neural network underlying respiration. Presumably, the tonic depolarizing drive provided by the release of EAAs in the brain stem-spinal cord preparation has been removed during the isolation of the medullary slice (Greer et al. 1991). This may result in complications when interpreting pharmacological data. There are two fundamental differences between the brain stem-spinal cord and medullary slice preparations. First, the membrane potential of neurons within the medullary slice are significantly more depolarized as compared with neurons in the brain stem-spinal cord preparation because of the higher (9 mM) potassium concentration needed to ensure rhythmicity in the slice. The increased potassium will depolarize pre- and post-synaptic terminals, actions which will augment the liberation of excitatory and inhibitory neurotransmitters as well as change the relative states of voltage sensitive ionic channels and the potassium reversal potential in the medullary slice as compared to the brain stem-spinal cord. Thus, the underlying substrate (ionic currents and basal neurotransmitter release) upon which drugs act will differ in the two preparations. Second, the two preparations contain different populations of viable neurons. In the case of the medullary slice, all but a limited portion of the medulla (pre-BötzC) has been removed. Thus, any extramedullary structures outside this region which could influence

respiratory rhythmogenesis will not play a role in the drug-induced response.

1.6 *Recapitulation of the thesis project outline*

To summarize, this study primarily centres on examining the actions of 5-HT and NA in modulating respiratory neural discharge generated by the neonatal rat medullary slice preparation. The effects of the two monoamines have generated a great deal of attention partly due to their proposed roles in mediating different sleep states and their involvement in sleep related respiratory distresses, such as OSAS (Conway et al. 1982). Therefore, the elucidation of the noradrenergic and the serotonergic effects on central respiratory drive may provide a possible insight to the neurochemical factors associated with such disorders. The effects of the two monoamines on the frequency of neural bursts and XII motoneuronal excitability were examined by directly affecting the rhythm generating centre and a subset of motoneurons controlling upper airway patency. The application of specific receptor agonists and antagonists was used to further determine the receptor subtypes responsible for the varying effects observed. Immunolabelling was used to determine whether 5-HT and catecholaminergic fibres are present within the rhythm generating centre and the hypoglossal motoneuron nuclei thus providing a morphological basis for the pharmacological effects observed.

CHAPTER 2

2. MATERIALS and METHODS

2.1 *Medullary slice preparation*

Experiments were performed using a medullary slice preparation that retains a functional respiratory network. Sprague-Dawley neonatal rats (0-4 days old) were anaesthetized with metofane, decorticated and the remaining vertebral column placed in a dissection chamber filled with 'artificial CSF solution' (for composition see below). The skull covering the brainstem was removed and a laminectomy was performed to expose the cervical spinal cord. The isolated brainstem-spinal cord was then pinned down, ventral surface upward, on a paraffin-coated block. The block was mounted in the vise of a vibratome bath (Pelco, VT 1000). The brainstem was serially microsectioned caudally in the transverse plane, starting from the pontomedullary junction (rostral medulla) to within ~200 μm of the rostral boundary of the pre-BötzC, as judged by the appearance of the inferior olive. A single transverse slice containing the pre-BötzC and more caudal reticular formation regions was then cut (500-600 μm thick), transferred to a recording chamber and pinned onto a Sylgard elastomer. The medullary slice was bathed in physiological solution similar to that used for the brain stem-spinal cord preparation, except for the potassium concentration, which was elevated to 9 mM. The bath contained a physiological solution consisting of (mM): 128 NaCl, 9.0 KCl, 1.5 $CaCl_2$, 1.0 $MgSO_4$, 24 $NaHCO_3$, 0.5 NaH_2PO4, and 30 D-glucose equilibrated with 95%O_2-5%CO_2 at 27°C

(pH=7.4).

2.2 *Drug application*

2.2.1 *Bath application of drugs.*

Dose-response data were derived by measuring the changes in frequency of respiratory XII nerve bursts in response to cumulative addition of increasing concentrations of drugs to the bathing medium (~10 ml bath), except during the assaying of 5-HT and NA's effects. Dose-response data for 5-HT and NA were derived from trials where a single dose of 5-HT or NA was added to the bathing medium. This was followed by either wash-out and a subsequent application of a different dose of 5-HT or NA, or the addition of a 5-HT or NA receptor antagonist. The accumulative administration of increasing doses of 5-HT or NA to the preparation's bathing medium produced a reduced response in comparison to what was observed with single doses (perhaps due to tachyphylaxis). Mean values for the period of respiratory nerve discharge (Fig. II-1B and Fig. II-5C) were calculated for 2-3 minutes pre- (control) and 5-10 minutes post-application of drugs to the bathing medium. The mean values for the period of respiratory drive nerve discharge (post application) of each experiment was compared to the control value (pre-drug application).

2.2.2 *Pressure injection of drugs.*

For focal application, drugs were delivered via pressure ejection (20 psi; Picospritzer, General Valve Co.) using either single- or double-barrelled micropipettes.

In the case of double barrelled electrodes, one electrode was filled with one of the following test drugs diluted in artificial CSF containing 9 mM K^+; 1 mM 5-HT, 1mM NA, 1 mM (R,S)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid hydrobromide (AMPA), and 1 mM R(-)2-(2,5-dimethoxy-4-iodophenyl) aminoethane hydrochloride (DOI.HCl). The second barrel was used to deliver either the vehicle solution (artificial CSF containing 9 mM K^+) or pontamine sky blue for the marking of ejection sites. Pipettes were mounted on a microdrive (Newport) and advanced (100-250 μm from the surface) into regions near either the nucleus raphé obscurus, the pre-BötzC, or the hypoglossal motoneuron pool. Injection volumes were determined to be less than 25 nl (<25 pmoles) by observing the fluid meniscus with a microscope equipped with a calibrated reticule. The vehicle injected at equal volumes did not modify the spontaneous respiratory rhythmic discharge. A fifteen minute interval between successive applications of drug was sufficient to allow for the wash-out of previously applied drug and a return to base-line respiratory rhythmic motor discharge. Injection sites were marked by iontophoretic deposition of dye. Slices were fixed in 4% paraformaldehyde, frozen and sectioned on a cryostat (40 μm), and counter-stained with neutral red. Camera lucida reconstructions were made to illustrate the position of the dye spots in the transverse plane of the medullary slice. With regards to the location of the region of interest, pontamine sky blue dye spots were utilized to identify injection sites and rapid responses following drug injections were an indication of regional proximity.

2.2.3 *Drugs applied.*

The specificity and characterization of the 5-HT receptor agonists and antagonists used in this study have been described by Peroutka et al. (1990). Drugs added to the *in vitro* bathing solution included: 5-HT; 8-OH-DPAT.HBr (R(+)-8-hydroxydipropylaminotetralin hydrobromide), a selective 5-HT_{1A} receptor agonist; DOI.HCl, a selective 5-HT_2 receptor agonist with a higher affinity for the 5-HT_{2A} receptor subtype; ketanserin tartrate, a selective 5-$HT_{1C/2}$ receptor antagonist; methysergide, a relatively non-selective 5-HT_1 and 5-HT_2 receptor antagonist; 1-(m-chlorophenyl)-biguanide HCl (m-CPBG), a 5-HT_3 receptor agonist.

Adrenergic agonists and antagonists added to the bathing medium included: NA bitartrate; phenylephrine HCl R(-), an α_1 receptor agonist; idazoxan HCl, an α_2 receptor antagonist and prazosin HCl, an α_1 receptor antagonist.

Dr. F. G. Boess from the Department of Pharmacology at the University of Alberta kindly provided the m-CPBG. 5-HT was obtained from Sigma, and all other compounds were purchased from Research Biochemicals International.

2.3 *Recording and analysis*

Recordings of cranial XII nerve activity were made with suction electrodes applied to the cut ends of XII roots. Signals were amplified, rectified, low passed filtered and recorded on computer via an analogue-digital converter (DigiData 1200, Axon Instruments) and data acquisition software (Axotape, Axon Instruments). Results were expressed as mean percentage $\pm$ standard deviation (relative to control) and any

differences were tested using paired difference student's t-test; significance was accepted at p values lower than 0.05. Programs for calculating frequency, amplitude and burst duration were written by Dr. Matt Wheatly and Sophie DeSerres. Robin Erickson, worked as a summer student in our laboratory for four months during 1994 and contributed significantly to the completion of the data analysis.

2.4 *Immunolabelling*

Tissues were fixed in 4% paraformaldehyde (overnight, 4°C) and cryoprotected with 30% sucrose. Subsequently tissues were frozen and sectioned (40 μm) on a cryostat (Jung CM3000).

Slices were thoroughly washed with phosphate buffered saline (PBS, pH 7.4) for 4-5 hours and incubated overnight with the primary antibody that recognizes 5-HT. The dilution of anti 5-HT (Cambridge Research Biochemicals, raised in goat) was 1:200. The primary antibody was diluted in PBS with 0.4% Triton X-100 (ICN Biomedicals Inc). Triton X-100 causes tissue membranes to permeabilize allowing the primary antibody to adequately bind onto all of the 5-HT epitopes in the tissue.

Tissues were incubated with a 1:200 dilution of biotinylated donkey anti-goat for 1 hour, and rinsed again with PBS to remove any unbound biotinylated anti-goat antibody. The epitope was visualized by incubating the tissues with Texas red conjugated to avidin (1:200 for 40 minutes, Amersham International, U.K.)

The immunofluorescence protocol pertaining to catecholamine detection was similar to that for 5-HT. Briefly, tissues were incubated in a 1:200 diluted rabbit anti-rat

TH (tyrosine hydroxylase) antibody (TE101- Eugene Tech International). TH primary antibody was then visualized by incubating tissues with a 1:200 diluted goat anti-rabbit secondary antibody, conjugated to FITC. All tissues were incubated as free floating sections at room temperature.

The sections were air dried, mounted on glass slides, coverslipped with Cytoseal (Stephens Scientific) and examined with a Zeiss epifluorescence microscope, equipped with filter combinations revealing red and green fluorescence.

To control for the specificity of the immunocytochemical reactions, sections were incubated as described above with alternating omission of primary antibodies. Nonspecific immunofluorescence was not detected in these control experiments.

CHAPTER 3[1]

3. RESULTS

3.1 *Effects of 5-HT on respiratory rhythm and XII nerve discharge*

5-HT administered to the media bathing *in vitro* medullary slice preparations produced a significant increase in the frequency of the rhythmic respiratory bursts and tonic discharge of the XII cranial nerves (10 of 10 cases; Fig. II-1A). The maximum response was observed with the addition of 30 μM, which caused an increase in the frequency of respiratory discharge ($37\pm16\%$; $p<0.05$) and burst duration ($58\pm11\%$; $p<0.05$) and a decrease in the amplitude ($16\pm9\%$; $p<0.05$) of XII motor nerve discharges. The 5-HT induced changes in respiratory burst frequency and XII nerve discharge were blocked by the application of the 5-HT receptor antagonist methysergide (40 μM). The addition of methysergide (40 μM) to the bathing medium on its own decreased the respiratory rhythm in two cases (8 and 11%) and increased it in three cases (6,7, and 8%) but overall there was no significant change ($0.4\pm9\%$; $n=5$; $p>0.05$). To delineate the site within the medullary slice where 5-HT was acting to increase respiratory rhythm frequency, focal pressure injections of 5-HT were performed. Injections of ~25 pmol 5-HT into regions previously identified as the pre-BötzC (Funk et al. 1994), caused an increase in the frequency of respiratory rhythmic discharge

---

[1]*Results presented in this chapter have been incorporated into a paper currently in press in Pflügers Archiv (Al-Zubaidy, Erickson & Greer, 1996).*

(22±14%; n=8; range=16-51%; $p<0.05$) which was blocked by bath application of methysergide (30 μM) (Fig. II-1C). A small increase in the tonic discharge of the XII nerve occurred in 2 of 8 cases in response to pressure injection of 5-HT into the pre-BötzC; presumably via activation of XII pre-motoneurons located within the ventrolateral medulla .

To determine if the increases in respiratory frequency caused by bath and focal applications of 5-HT could be induced by increasing the endogenous release of 5-HT, regions of the medullary slice containing the nucleus raphé obscurus were activated via focal pressure ejection of the glutamate receptor agonist AMPA or 5-HT. Pressure injection of either ~25 pmol AMPA (34±15%; n=4; range=13-49%; $p<0.05$) or 5-HT (33±5%; n=8; range=23-38%; $p<0.05$) into the nucleus raphé obscurus produced an increase in respiratory burst frequency. Tonic discharge of XII nerve activity also resulted from AMPA or 5-HT mediated stimulation of nucleus raphé obscurus in 3 of 4 and in 7 of 8 cases, respectively. The AMPA induced changes in respiratory frequency and tonic XII nerve discharge were partially antagonized by application of methysergide (30-40 μM) to the bathing medium in each case. Whereas bath application of methysergide completely blocked any effects of pressure injecting 5-HT into the nucleus raphé obscurus (Fig. II-2).

It was not clear which receptor subtypes were responsible for the 5-HT mediated increase of respiratory frequency. Bath application of 5-$HT1_A$ receptor agonist 8-OH-DPAT, (30 μM; n=9), resulted in a 1.1±12.5% ($p>0.05$), 15±10% ($p>0.05$) and 6±6% ($p>0.05$) change in XII nerve discharge respiratory frequency, amplitude and duration, respectively. Similarly, neither of the 5-HT_2 agonist DOI.HCl. R(-), (30 μM;

12.1±18.5%; n=6), neither the 5-HT_3 receptor agonist m-CPBG (20 μM; 2.0±4.4%; n=3) caused a significant change in respiratory discharge frequency.

The 5-HT induced changes in XII nerve discharge were mimicked by the bath application of the 5-HT_2 receptor agonist DOI.HCl (5 μM; n=4) (Fig. II-3B). DOI.HCl caused an increase in XII nerve tonic discharge and burst duration (47±13%; $p<0.05$) and a decrease in burst amplitude (11±19%; $p>0.05$). Similarly, pressure ejection of 5-HT (Fig. II-3A) into the HMN caused an increase in XII nerve tonic discharge and burst duration (64±24%; $p<0.05$) and a decrease in burst amplitude (8±11%; $p<0.05$). In the presence of 5-HT_2 receptor antagonist ketanserin (30 μM), the DOI.HCl induced increase in burst duration was (3±3%; $p>0.05$) and (10±4%; $p<0.05$) when added to the bath or pressure injected (Fig. II-3C), respectively. The DOI.HCl induced tonic discharge was also antagonized by bath application of ketanserin (30 μM).

3.2 *Effects of NA on respiratory rhythm and XII nerve discharge*

NA administered to the media bathing *in vitro* medullary slice preparations typically caused a dose-dependant decrease in the frequency of respiratory neural discharge (maximum of 60±15% at 100 μM; n=8; $p<0.05$). However, in 2 of 8 cases tested, there was an increase (28% and 32%) in respiratory neural discharge frequency in response to bath application of lower doses (500 nM) of NA. The maximum response was observed with the addition of 100 μM NA to the bathing medium which caused a decrease in the frequency (60±15%; $p<0.05$) and increases in burst duration (13±8%; $p<0.05$) and amplitude (13±3%; $p<0.05$) of XII motor nerve discharge.

Fig. II-1 5-HT increases respiratory discharge frequency in the medullary slice preparation.

A) Left drawing depicts the neonatal rat medullary slice preparation showing arrangement of recording electrode on hypoglossal (XII) cranial roots. Right panel shows rectified and filtered signals from suction electrode recordings of inspiratory motor discharge from the hypoglossal cranial nerve (XII). Top trace shows control recording. Traces 2-4 show the response of XII nerve discharge after the addition of 30 μM 5-HT to the bathing medium. Final trace shows recording after wash-out of 5-HT from the bathing medium. 5-HT caused a reversible increase in the spontaneous respiratory burst frequency and induced XII nerve tonic discharge.

B) Bar graph showing population dose-response data ($n=10$) for the changes in frequencies of respiratory rhythmic discharges after administration of 5-HT to the medium bathing the medullary slice. 5-HT-induced increases in respiratory frequency were antagonized by 40 μM methysergide. Respiratory burst frequencies are plotted as fraction of control frequencies versus concentration of 5-HT added to the bathing media. Asterisks indicate doses which produced a significant ($p<0.05$) change from control values.

C) Recording of XII nerve discharge while 5-HT was applied focally to the region of the nucleus ambiguus in the ventrolateral medulla via pressure ejection. Time of pressure injections of 5-HT shown by arrows. During the break in the recording (shown by the third arrow), 30 μM methysergide was added to the bathing medium. Subsequent pressure injection of 5-HT into the region of the pre-BötzC (fourth arrow) did not cause a significant perturbation of respiratory frequency.

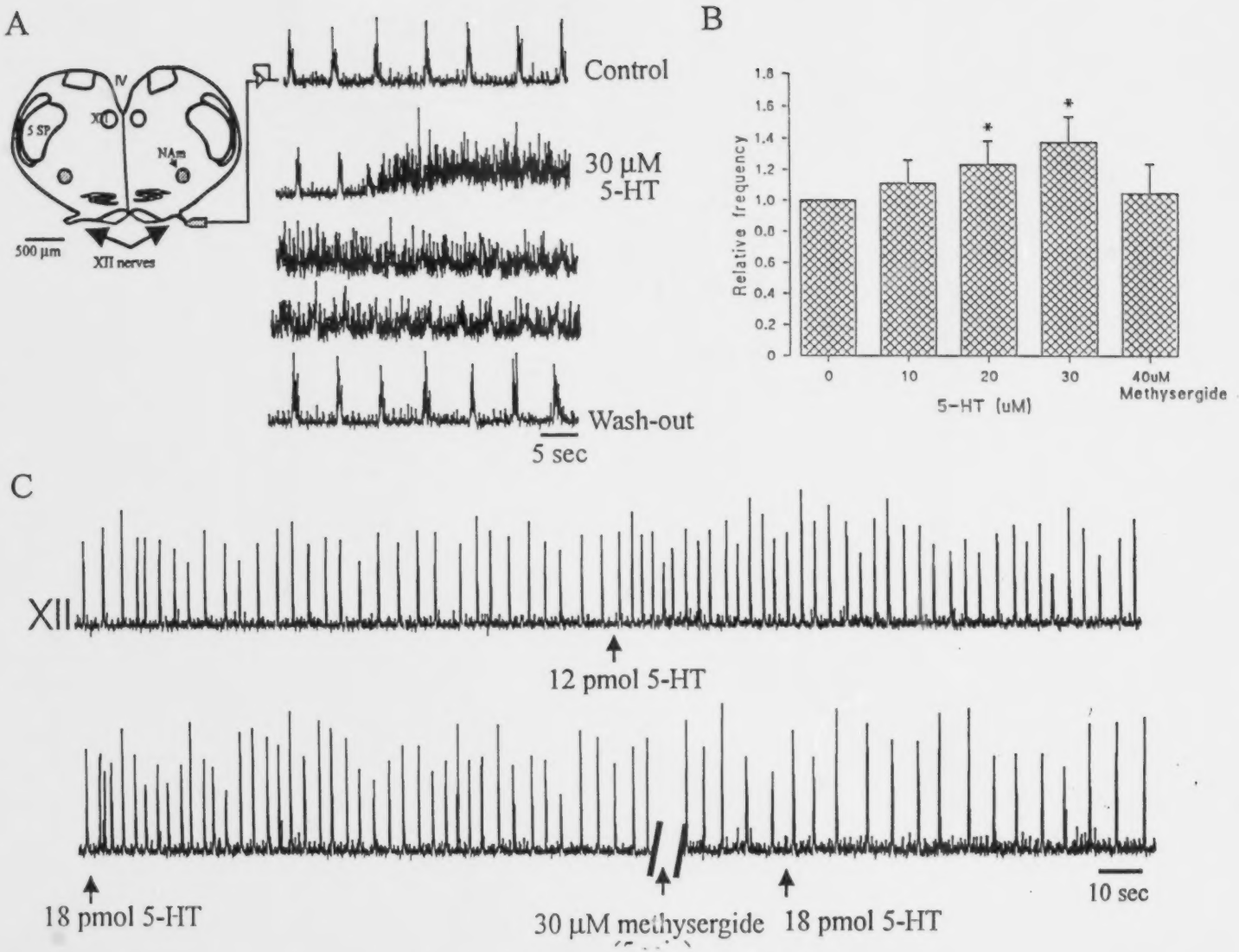

A
IV
5 SP
XII
NAm
500 µm
XII nerves
Control
30 µM
5-HT
Wash-out
5 sec
B
Relative frequency
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
10
20
30
40uM
Methysergide
5-HT (uM)
C
XII
12 pmol 5-HT
18 pmol 5-HT
30 µM methysergide
18 pmol 5-HT
10 sec

Fig. II-2 Activation of neurons within the nucleus raphé obscurus (NRO) via focal pressure injection of the EAA agonist AMPA caused a reversible increase in respiratory discharge frequency and tonic discharge of XII nerve. AMPA was injected into the RO at times shown by arrows. During the break in the recording (shown by fourth arrow), 30 μM methysergide was added to the bathing medium. Subsequently, the increase in respiratory frequency caused by AMPA injection into RO (fifth arrow) was partially antagonized in the presence of methysergide.

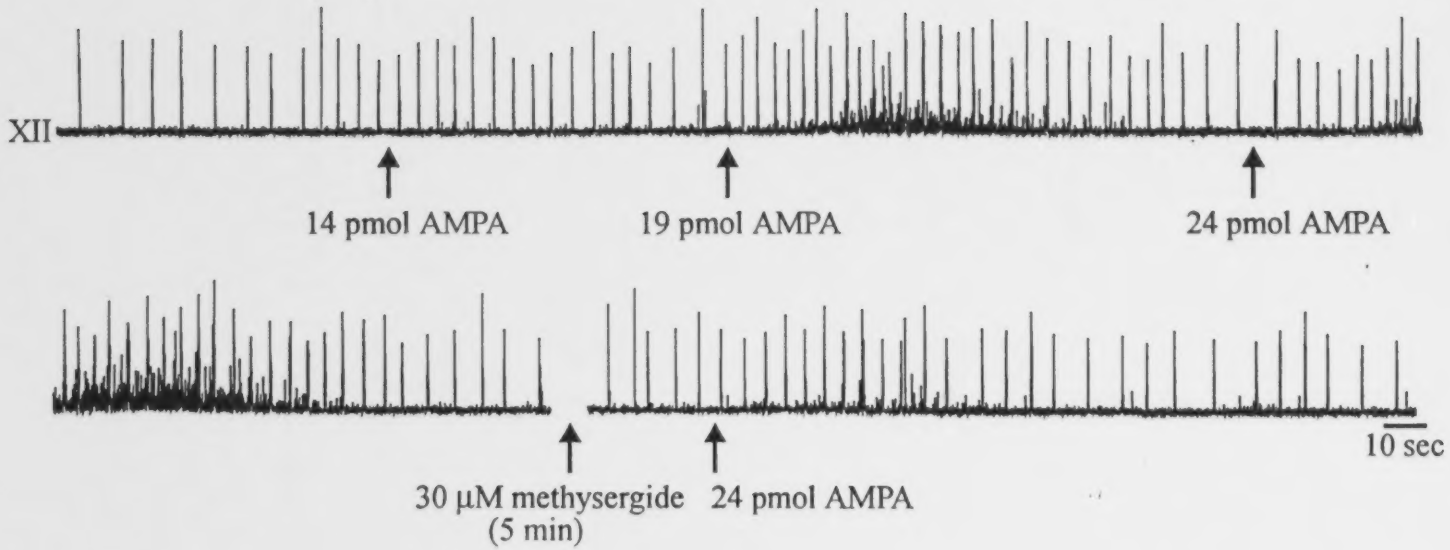

XII
14 pmol AMPA
19 pmol AMPA
24 pmol AMPA
10 sec
30 μM methysergide
(5 min)
24 pmol AMPA

Fig. II-3 5-HT receptor mediated increase of XII nerve tonic discharge in the medullary slice preparation.

A) Pressure injection of 5-HT into the XII motor nucleus caused tonic discharge of XII nerve (first arrow). This effect was blocked (third arrow) by adding 40 μM methysergide to the bathing medium (at time shown by second arrow).

B) Tonic discharge of XII nerve discharge was caused by the addition of the 5-HT_2 receptor agonist DOI (5 μM) to the bathing medium. This effect was antagonized by subsequent application of the 5-HT_2 receptor antagonist ketanserin (30 μM) to the bathing medium.

C) Focal application of DOI via pressure injection into the XII motor nucleus also caused tonic discharge of XII nerve discharge which was antagonized by bath application of ketanserin (30 μM).

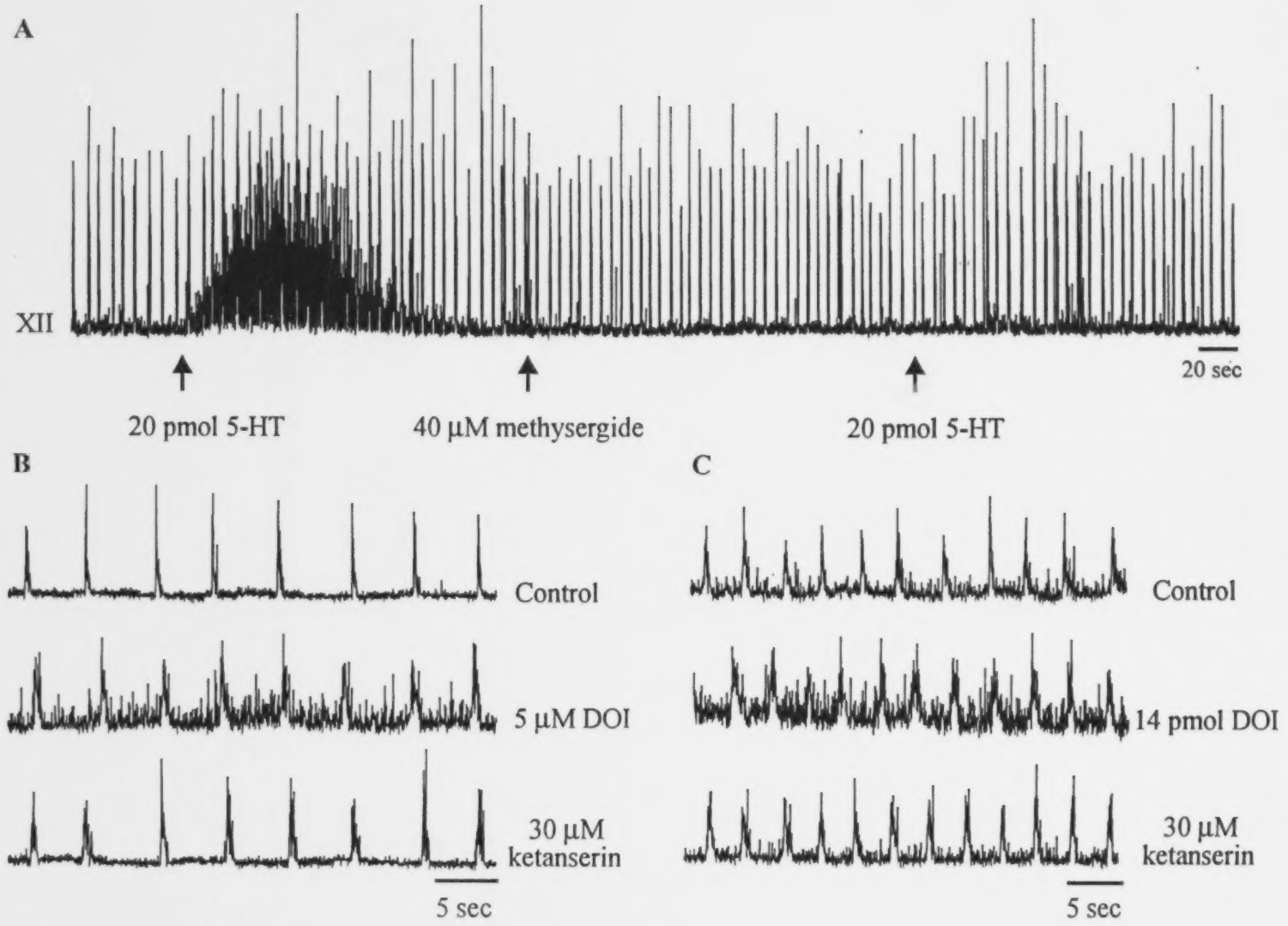
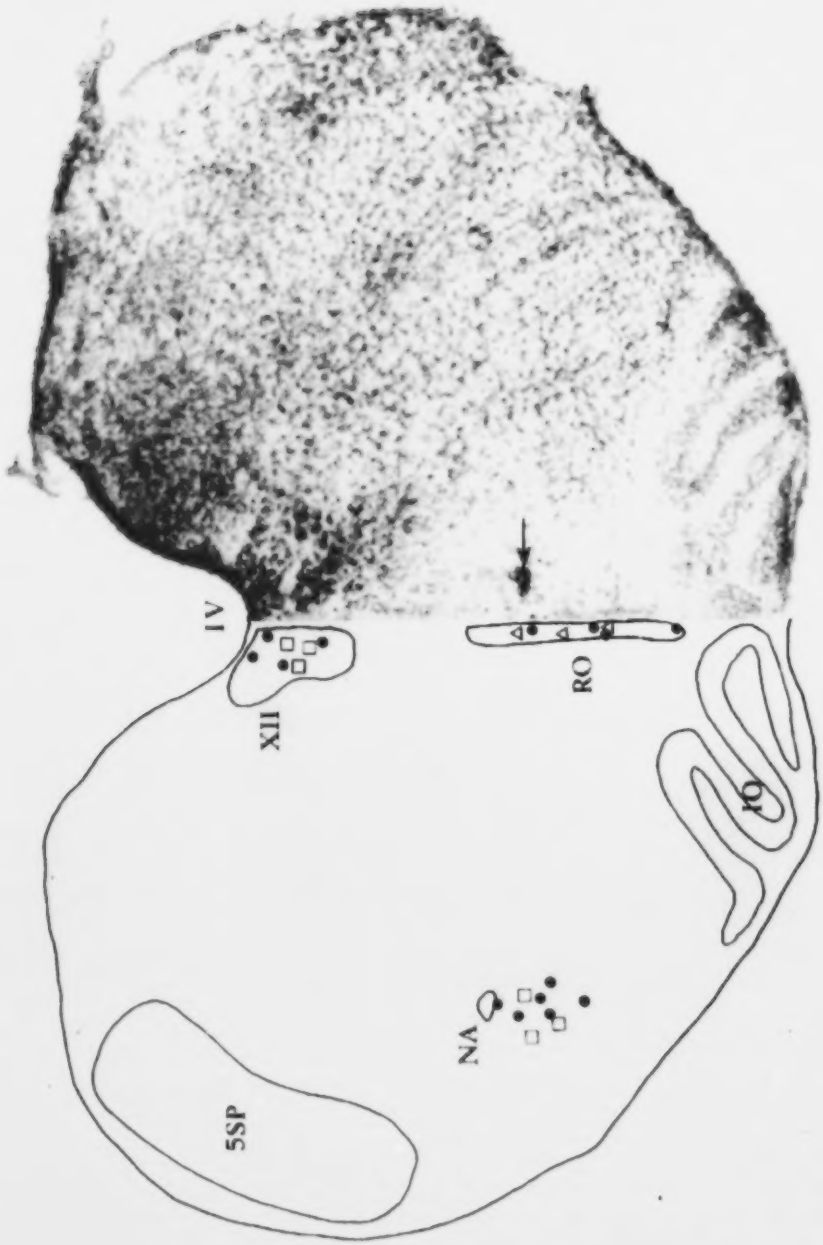

A
XII
20 sec
20 pmol 5-HT
40 µM methysergide
20 pmol 5-HT
B
Control
5 µM DOI
30 µM
ketanserin
5 sec
C
Control
14 pmol DOI
30 µM
ketanserin
5 sec

Fig. II-4 Location of pressure injection sites in the pre-BötzC, XII motor nucleus and nucleus raphé obscurus. Initial experiments were performed with single-barrel electrodes while latter experiments were performed using double-barrel electrodes for deposition of dye spots. Left side shows photomicrograph of medullary transverse section (40 μM thick) with dye spot (arrow) in the region of the RO and the right side shows a schematic of section showing relevant landmark nuclei and the reconstruction of dye spot positions. The following symbols are used to mark the sites where drugs were pressure injected and a dye spot was recovered. Filled circles show sites of 5-HT pressure injections into the 3 nuclei, squares show NA injection sites in the pre-BötzC and XII motor nucleus, and triangles show sites of AMPA injection into the raphe obscurus. No attempt was made to delineate the rostrocaudal distribution of effective injection sites within the medullary slice.

Abbreviations: 5SP, spinal trigeminal nucleus; NA, nucleus ambiguus; XII, hypoglossal motoneuron pool; IO, inferior olive; RO, raphé obscurus.


IV
XII
RO
IO
NA
5SP

NA induced perturbations of respiratory discharge frequency were reversed upon bath application of the α_2 adrenergic receptor antagonist idazoxan.HCl (2 μM). Idazoxan.HCl (2 μM), when added alone to the tissue's bathing medium, did not cause a significant increase in respiratory frequency ($1.1\pm3.5\%$; $n=3$) (Fig. II-5A).

Pressure ejecting ~25 pmol of NA into the pre-BötzC resulted in a decrease of the frequency of respiratory neural discharge ($22\pm11\%$; $n=3$; range=9%-31%; $p<0.05$), which was reversed upon wash-out (Fig. II-5B).

Bath application of NA resulted in the tonic discharge of XII cranial nerves (8 of 8 cases). The tonic XII nerve discharge was abolished upon application of α_1 adrenergic receptor antagonist prazosin HCl (1 μM) to the bathing medium and mimicked by bath application of the α_1 adrenergic receptor agonist phenylephrine HCl (500 nM) (Fig. II-5D). Pressure ejection of NA into the hypoglossal motoneuron pool caused tonic discharge of XII nerve activity in 4 of 4 cases, as well as increases in burst duration ($26\pm15\%$; $p<0.05$) and amplitude ($15\pm8\%$; $p<0.05$).

Fig. II-5 NA receptor mediated decreases in respiratory discharge frequency and inducement of XII nerve tonic discharge in the medullary slice preparation.

A) Addition of NA to the bathing medium caused a depression of respiratory discharge frequency and an increase in tonic XII nerve discharge. The decrease in respiratory discharge frequency was antagonized by the α_2 receptor antagonist idazoxan (2 μM).

B) Bar graph showing population dose-response data (n=8) for the changes in frequencies of respiratory rhythmic discharges after administration of NA to the bathing media. The NA induced decreases in respiratory frequency were antagonized by idazoxan (2 μM). Respiratory burst frequencies are plotted as fraction of control frequencies versus concentration of NA added to the bathing media. Asterisks indicate doses which produced a significant ($p<0.05$) change from control values.

C) XII nerve discharge in response to NA applied focally to the region of the nucleus ambiguus in the ventrolateral medulla via pressure ejection. Time of pressure injection of NA shown by arrow.

D) Bath application of the α_1 receptor agonist phenylephrine HCl (500 nM) also caused tonic discharge of the XII nerve, which was blocked by the α_1 adrenergic receptor antagonist prazosin (1 μM).

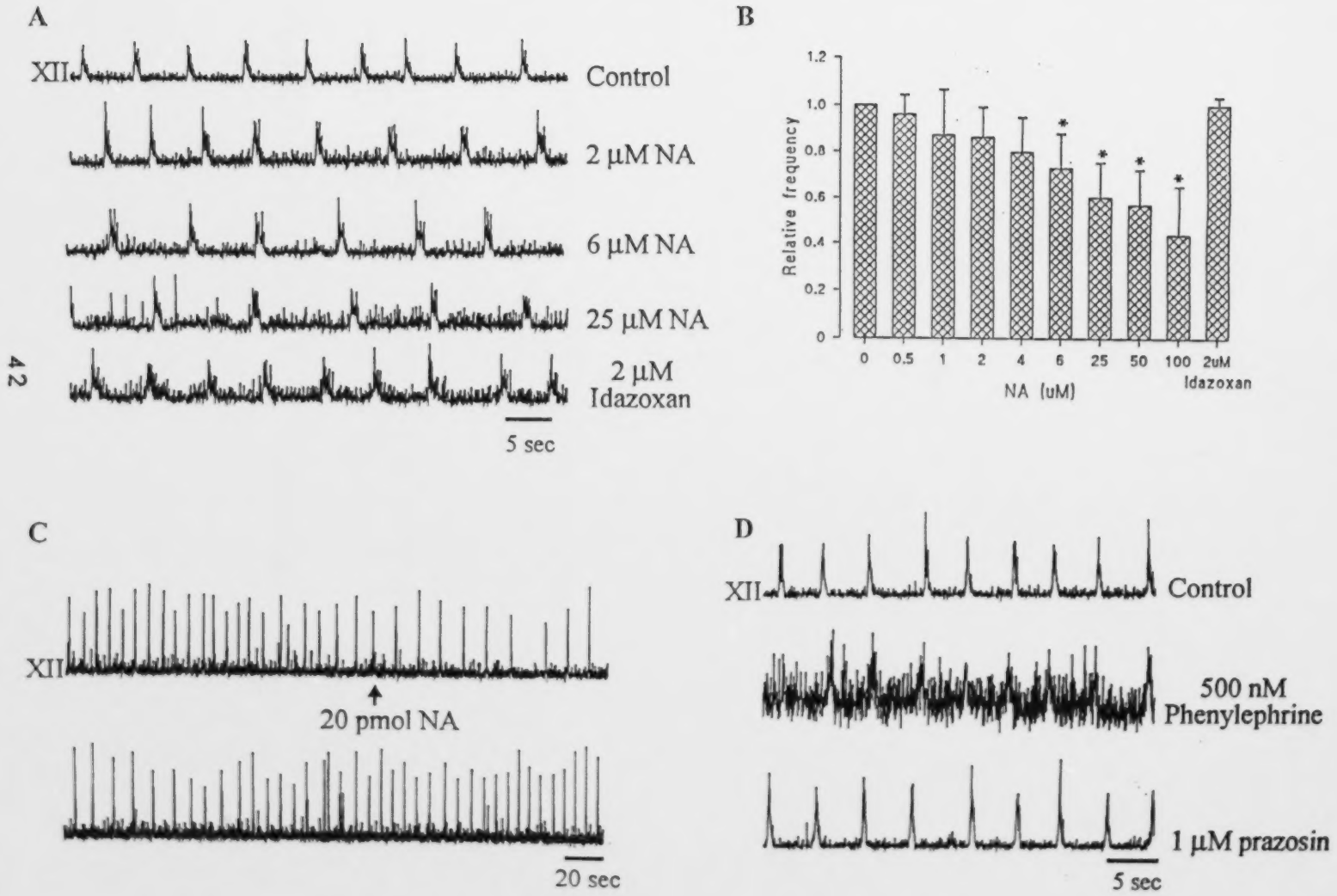

A
XII
Control
2 μM NA
6 μM NA
25 μM NA
2 μM
Idazoxan
5 sec
B
Relative frequency
1.2
1.0
0.8
0.6
0.4
0.2
0
0 0.5 1 2 4 6 25 50 100 2uM
NA (uM)
Idazoxan
C
XII
20 pmol NA
20 sec
D
XII
Control
500 nM
Phenylephrine
1 μM prazosin
5 sec

Fig. II-6 A series of photomicrographs demonstrating the presence of serotonergic immunopositive fibres within specific medullary regions.

A) A schematic diagram showing a transverse section of the medullary slice preparation with boxes illustrating regions from which photomicrographs are taken.

B) Photomicrographs showing serotonergic immunopositive fibres present in the region of the proposed rhythm generating centre (pre-BötzC). Serotonergic immunopositive fibres were consistently observed in all of the animals examined (n=4). Bar: 20 μm.

C) Photomicrographs showing serotonergic immunopositive fibres present in the region of the hypoglossal motoneuron pool. Bar: 20 μm.

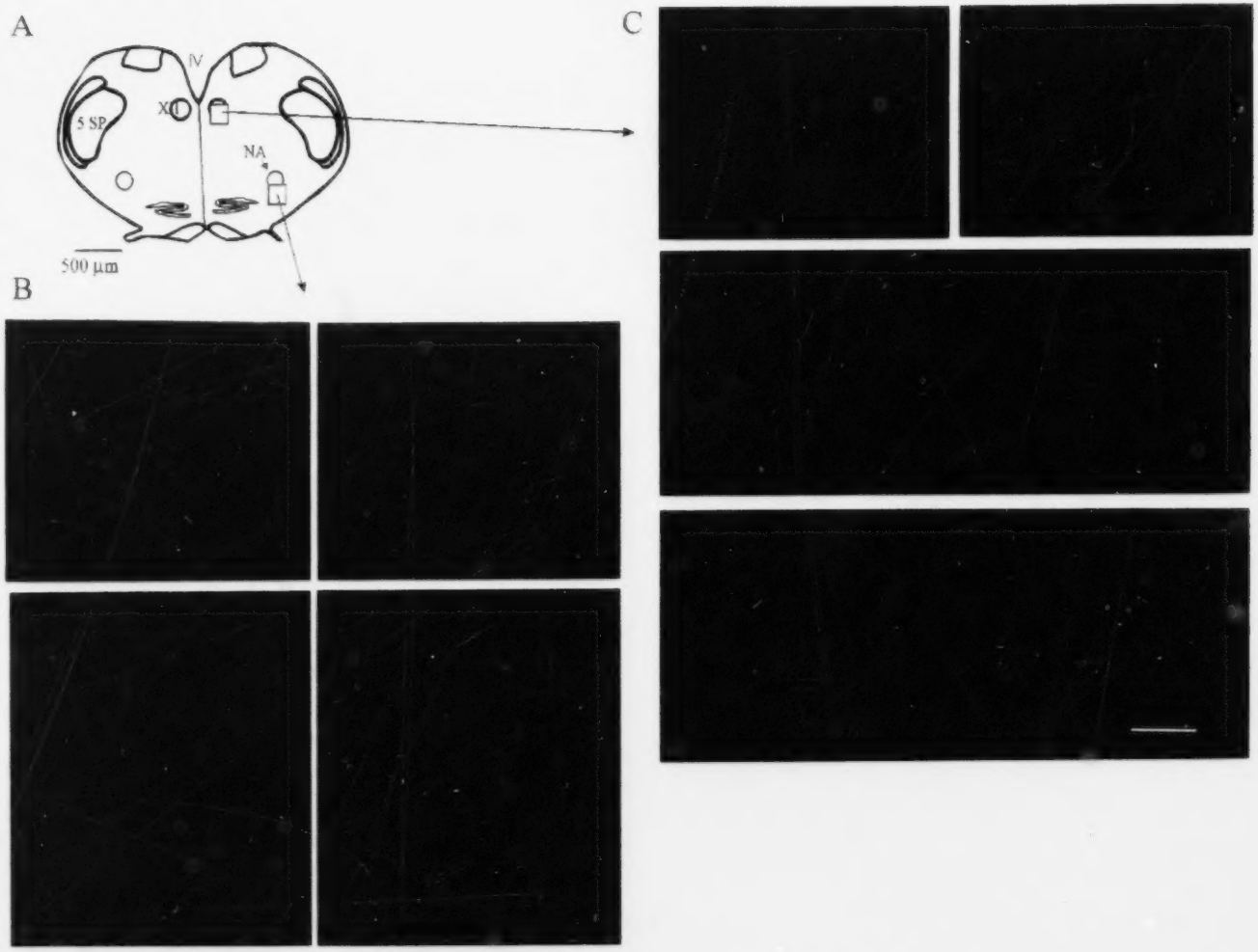
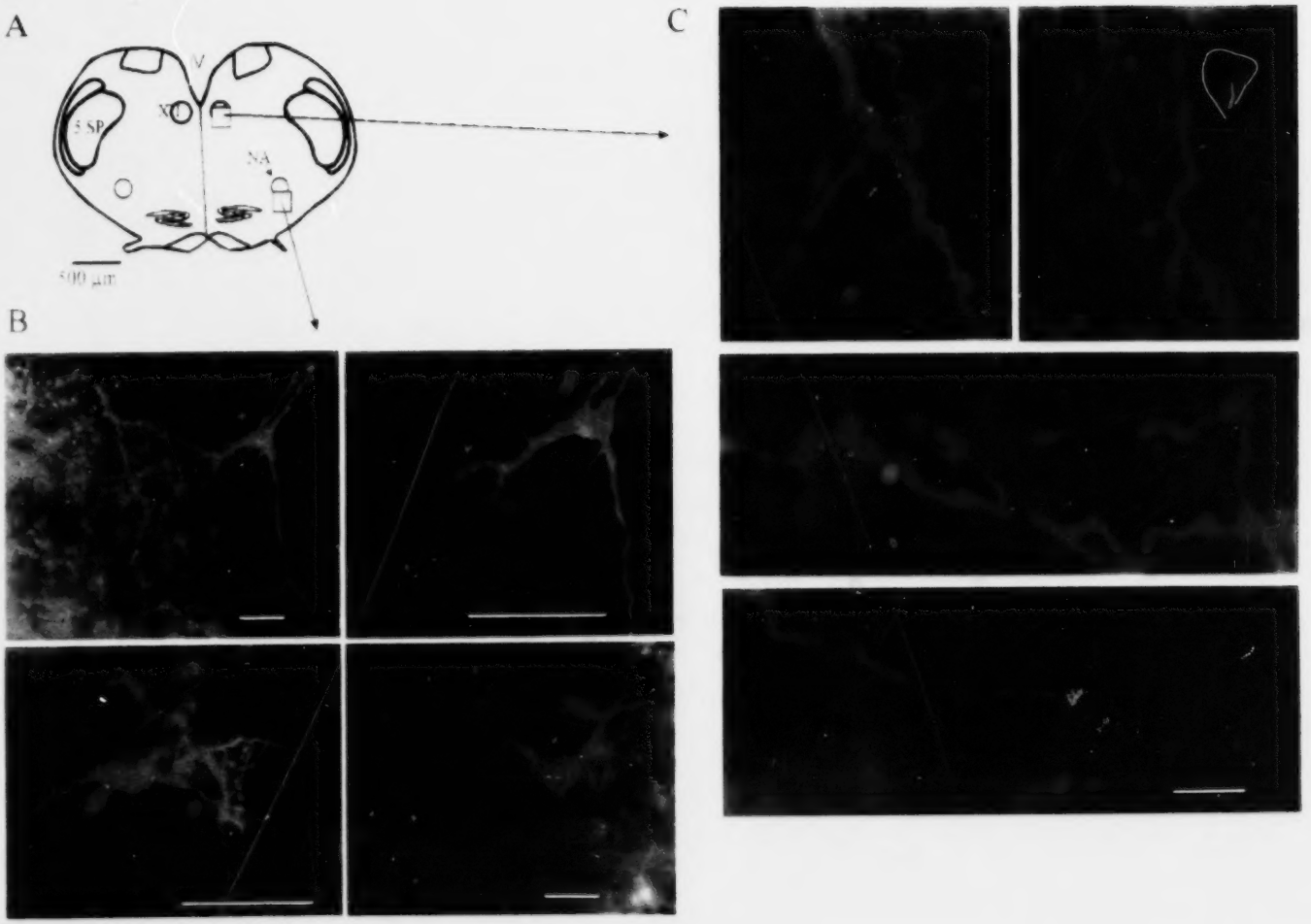

A
IV
5 SP
XII
NA
500 µm
B
C

Fig. II-7 A series of photomicrographs demonstrating the presence of tyrosine hydroxylase immunopositive cell bodies and fibres within specific medullary regions.

A) A schematic diagram showing a transverse section of the medullary slice preparation with boxes illustrating regions from which photomicrographs are taken.

B) Photomicrographs showing tyrosine hydroxylase immunopositive cell bodies and fibres present in the region of the proposed rhythm generating centre (pre-BötzC). Tyrosine hydroxylase immunopositive fibres were consistently observed in all of the animals examined (n=4). Bar: 60 μm.

C) Photomicrographs showing tyrosine hydroxylase immunopositive fibres present in the region of the hypoglossal motoneuron pool. Bar: 20 μm.


A
V
5 SP
XII
NA
500 μm
B
C

CHAPTER 4

4. DISCUSSION

This study demonstrates the following: (i) 5-HT and NA, acting in the region of proposed respiratory rhythm generating centres (pre-BötzC), stimulate and depress, respectively, the frequency of respiratory burst discharge *in vitro;* (ii) 5-HT and NA induce tonic XII cranial nerve discharge; (iii) The endogenous release of 5-HT from the nucleus raphé obscurus within the medullary slice causes an increase in respiratory discharge frequency and XII nerve discharge; (iv) The receptor subtype responsible for inducing the 5-HT increase in the respiratory related neural discharges was not determined, however, activation of 5-$HT_{1C/2}$ receptor is responsible for augmentation of XII nerve tonic discharge activity; v) The receptor subtypes accountable for mediating the noradrenergic-mediated decrease in respiratory frequency and the augmentation of XII nerve discharge amplitude are α_2 and α_1 -adrenoreceptors, respectively. (vi) Immunopositive nerve fibres, staining for 5-HT and TH are present in regions of the pre-BötzC and the HMN.

4.1 *5-HT mediated increases in respiratory frequency*

Morin et al. (1990a), utilizing the neonatal rat brainstem-spinal cord *in vitro* preparation, have shown that application of 5-HT to the medium bathing the medulla causes an increase in the frequency of respiratory bursts. In the present study, we wished

to elaborate on these findings by directly assaying the effects of exogenously applied and endogenously released 5-HT within the region of respiratory rhythm generating centres (pre-BötzC). We have shown that 5-HT induces an increase in respiratory frequency by acting directly within regions of the ventrolateral medulla encompassing the pre-BötzC. The previous studies of Morin et al. (1990a) suggest that the 5-HT mediated increase in respiratory frequency is being mediated via activation of 5-HT_{1A} receptors. Further support for involvement of 5-HT_{1A} receptors in affecting respiratory frequency comes from intracellular recording studies from pentobarbital anaesthetized cats (Lalley et al. 1994). We did not find any evidence for the involvement of another receptor subtype mediating 5-HT induced increases in respiratory frequency, although, for reasons that are not clear, we could not generate a significant increase in respiratory frequency in the medullary slice with the 5-HT_{1A} receptor agonist 8-OH-DPAT. It may be that the regions affected by 8-OH-DPAT in the brain stem-spinal cord preparation and the decerebrate, anesthetized cat are not contained or functional within the medullary slice preparation.

The effects of exogenous application of 5-HT were also produced by the endogenous release of 5-HT. The increase in the frequency of respiratory discharge in response to chemical stimulation of the nucleus raphé obscurus with AMPA and 5-HT, illustrates that there are functional projections within the medullary slice between the nucleus raphé obscurus and the pre-BötzC. The majority of the increase in respiratory frequency caused by the pressure injection of AMPA was antagonized by methysergide, suggesting that the endogenous release of 5-HT was involved. The remaining excitation may have been mediated by the actions of two neuromodulators which stimulate

respiratory discharge *in vitro*, substance P (Yamamoto et al. 1992) and/or TRH (Greer et al. 1995a), both of which are localized within raphé neurons (Törk, 1985). A similar non-5-HT mediated component of the excitation of phrenic motoneurons is evoked by stimulation of the nucleus raphé obscurus in the anesthetized cat (Lalley, 1986).

Pressure injection of either AMPA or 5-HT into the nucleus raphé obscurus was effective in producing perturbations of respiratory rhythmogenesis. The excitatory actions of 5-HT application within the nucleus raphé obscurus may seem puzzling in light of previous reports that at least a subpopulation of neurons within the medullary raphé neurons are inhibited by 5-HT, presumably via autoreceptors on 5-HT containing neurons (Clement and McCall, 1991). However, other studies involving pressure injection of 5-HT *in vivo* have shown a 5-HT mediated excitation of neuronal activity within the nucleus raphé obscurus (Dreteler et al. 1991). Therefore, we propose that both AMPA and 5-HT act to depolarize a sub-population of neurons within the nucleus raphé obscurus of the medullary slice which in turn results in the endogenous release of 5-HT. The rhythmically active medullary slice should be amenable for studies utilizing intracellular recordings from neurons within the nucleus raphé obscurus, XII motor nucleus and the pre-BötzC to further address issues pertaining to the interactions of these three nuclei.

4.2 5-HT mediated increases in tonic XII nerve discharge

Our results show a clear augmentation of XII nerve tonic discharge by either the exogenous application, or endogenous release from the nucleus raphé obscurus, of 5-HT. These effects were antagonized and mimicked by $5\text{-HT}_{1C/2}$ receptor antagonists and

agonists, respectively. It was not possible to clearly ascertain from our whole nerve recordings which, if any, of the 5-HT induced tonic discharge was due to depolarization of XII motoneurons receiving inspiratory drive. Moreover, intracellular analysis will be necessary to determine if the changes in burst duration and motor discharge amplitude are due to 5-HT mediated changes in pre and/or post-synaptic events. However, there was certainly no evidence for a total depression of inspiratory drive transmission similar to that reported by Morin (1990b, 1993) for the brain stem-spinal cord *in vitro* preparation. Rather, our results are in agreement with reports of intracellular recording studies in non-oscillating medullary slices which showed a 5-HT mediated excitation of XII motoneurons (Berger et al. 1992). Similarly, *in vivo* cat studies have shown that XII motoneuron excitability is increased via focal application of 5-HT and decreased by lowering the endogenous 5-HT levels in the XII motoneuron pool (Kubin et al. 1992, 1994). Thus, we feel that 5-HT acts to augment rather than depress the level of XII motoneuron excitability.

4.3 NA mediated decreases in respiratory frequency and increases in tonic XII nerve discharge

Results from the present study illustrate that the NA mediated depression of respiratory frequency reported previously with *in vitro* (Errichidi et al. 1991) and *in vivo* (Bolme & Fuxe 1973) studies is due at least in part to actions mediated via α_2 adrenergic receptors located in regions encompassing the pre-BötzC. The finding that NA caused tonic discharge of XII nerve differs from that of Errchidi et al. (1991) who report that

the addition of NA never affected XII motor nerve discharge when added to the medium bathing the brain stem-spinal cord preparation. However, our findings are in agreement with the results reported for the juvenile mouse *in vitro* preparation by Funk et al. (1994) and from intracellular recordings of hypoglossal motoneurons from non-oscillating neonatal rat medullary slices (Parkis et al. 1994). Previous studies have not addressed the issue of which receptor subtype was involved in the NA-induced tonic activation of XII motoneurons, but our results suggest that α_1 adrenergic receptor activation is responsible in the neonatal rat medullary slice preparation.

Thus, an increase in the endogenous release of NA within the pre-BötzC and the XII motor nuclei would tend to decrease respiratory frequency and increase XII nerve discharge, respectively. Whether or not there are such state-dependent changes in NA release within these regions is uncertain. The fact that the discharge rate of neurons within the locus coeruleus is depressed during sleep would suggest a decrease in NA levels (Jones, 1991). However, there have been reports of elevated brain and systemic NA levels associated with sleep apnea and SIDS, perhaps as a result of increased activity of pontomedullary catecholaminergic neurons associated with cardiorespiratory control (Bhat et al. 1983; Rodriguez et al. 1987).

4.4 5-HT and NA containing nerve fibres within the pre-BötzC and XII motoneuron pool.

4.4.1 *5-HT immunofluorescence*

Origins of 5-HT containing projections to the ventral respiratory group have been previously investigated (Holtman 1990). Immunohistochemical studies showed that 5-HT

terminals located close to inspiratory neurons in the rostral portion of the ventral respiratory group (rVRG) originate from the caudal raphé bodies; nucleus raphé pallidus (B1), nucleus raphé obscurus (B2) and nucleus raphé magnus (B3). 5-HT containing nerve terminals are also widespread within the HMN (Aldes et al. 1988). 5-HT containing terminals within the hypoglossal motoneuronal pool originate from the nucleus raphé obscurus and ventral medullary reticular 5-HT containing cells (nucleus raphé magnus) (Aldes et al. 1989). Our immunolabelling demonstrated the presence of 5-HT containing nerve fibres specifically within the region of the respiratory rhythm generating centre and the subpopulation of hypoglossal motoneurons contained within the medullary slice preparation.

4.4.2 *Tyrosine hydroxylase immunofluorescence*

Several studies have demonstrated the presence of noradrenergic terminals arising from the C1 cell group (ventrolateral medulla) are intermingled within inspiratory and expiratory neuron populations (Pilowski et al. 1994, Sun et al. 1994). The hypoglossal nucleus also receives noradrenergic innervation from the locus subcoerulus (A7), locus coeruleus (A6) and the pontine A5 region (Aldes et al. 1992). There are also catecholaminergic axonal processes innervating the HMN originating from the C2 region (caudal part nucleus solitarius - NTS and dorsal vagal nucleus) (Dahlström and Fuxe 1964).

Our immunolabelling demonstrates that catecholaminergic containing fibres are located within the subpopulation of XII motoneurons contained within the medullary slice

and in the vicinity of the proposed respiratory rhythm generating centre.

4.5 Implications of state-dependent changes and the balance of monoamine levels

Two fundamental disorders associated with neonatal breathing are OSAS and sudden infant death syndrome (SIDS). There is a strong correlation between the occurrence of these anomalies and different sleep states (Scher et al. 1992). The fact that altered levels of monoamines are released within the CNS during sleep suggests that transient imbalances of monoaminergic inhibitory and excitatory drive to respiratory rhythm generating centres and/or upper airway musculature could contribute to the pathologies. This hypothesis, at least as it pertains to cranial motoneurons, is supported by the recent study of Kubin et al. (1994) showing that 5-HT levels are depressed in the XII nucleus during carbachol-induced REM sleep-like atonia. Clearly, further *in vivo* measurements of state-dependent changes of catecholamine levels within brainstem respiratory nuclei are needed to provide more direct information regarding the overall balance of 5-HT and NA levels within the pre-BötzC and cranial motoneuron populations. Results from the present study suggest: i) a decrease in the ratio of 5-HT / NA release within the pre-BötzC will result in a net depression of central respiratory drive, and ii) a decrease of the tonic release of either transmitter within the XII nucleus will result in a disfacilitation of motoneuron excitability and the subsequent potential for upper airway obstruction.

4.6 *Suggestions for future work*

The neonatal slice preparation offers a relatively simple, yet direct, means for examining the neurochemical influences on respiratory related neural discharges and motoneuronal activity. Thus, one can obtain information regarding how various neuromodulators specifically effect the function of the proposed respiratory rhythm generating centre (pre-BötzC) and a subset of motoneurons controlling upper airway patency (XII motoneurons). This includes the identification of receptor subtypes responsible for eliciting the different effects observed. The medullary slice and brain stem spinal cord preparations are also viable when isolated from fetal animals from the time of the onset of respiratory rhythmogenesis (embryonic day 17; gestation period=21 days) onwards (Greer et al. 1992b). Therefore, a developmental profile of the onset and degree of modulation induced by various neurochemicals can be assayed pre- and postnatally with this preparation.

The medullary slice preparation contains different classes of respiratory neurons, including inspiratory, expiratory, and pacemaker-like cells. Thus, one could perform intracellular recordings (whole-cell techniques) in conjunction with pharmacological studies to examine the role of various neurochemicals in modulating the neuronal properties and firing patterns of each class of respiratory neuron. Moreover, by combining intracellular fills with neurobiotin or lucifer yellow and immunolabelling, one could determine the distribution of neurotransmitters contained in terminals within the immediate vicinity of a given cell. With the increasing availability of markers for neurotransmitter receptors, one could also combine the intracellular fills with receptor

labelling to determine the spatiotemporal distribution of receptors on different classes of respiratory neurons.

In summary, *in vitro* preparations derived from fetal and neonatal rats offer the opportunity to perform studies which would be difficult or impossible to perform in whole animal models. Thus, the use of these perinatal *in vitro* preparations should continue to be a powerful experimental model which can be used to complement in *vivo* studies towards understanding the neurochemical control of mammalian perinatal breathing.

REFERENCES

ALDES, L.D., CHRONISTER, R.C., MARCO, L.A., HAYDOCK, J.W. and THIBAULT J. (1988). Differential distribution of biogenic amines in the hypoglossal nucleus of the rat. *Experimental Brain Research* **73**:305-314.

ALDES, L.D., MARCO, L.A. and CHRONISTER, R.B. (1989). Serotonin-containing axon terminals in the hypoglossal nucleus of the rat. An immuno-electromicroscopic study. *Brain Research Bulletin* **23**:249-256.

ALDES, L.D., CHAPMAN M.E., CHRONISTER, R.B. and HAYDOCK, J.W. (1992). Source of noradrenergic afferent to the hypoglossal nucleus in the rat. *Brain Research Bulletin* **29**:931-942.

ARITA, H. and OCHIISHI, M. (1991). Opposing effects of 5-hydroxytryptamine on two types of medullary inspiratory neurons with distinct firing patterns. *Journal of Neurophysiology* **66**:285-292.

ARITA, H., SAKAMOTO, M., HIROKAWA, Y. and OKADO, N. (1993). Serotonin innervation patterns differ among the various medullary motoneuronal groups involved in upper airway control. *Experimental Brain Research* **95**:100-110.

BERGER, A.J., BAYLISS, D.A. and VIANA, F. (1992). Modulation of neonatal rat hypoglossal motoneuron excitability by serotonin. *Neuroscience Letters* **143**:164-168.

BHAT, A.M., SCANLON, J.W., LAVENSTEIN, B., CHUANG, L. and KAROUM, F. (1983). Cerebrospinal fluid concentration of biogenic amine metabolites in idiopathic apnea of prematurity. *Biology of the Neonate* **43**:16-22.

BOAKES, R.J., BRADLEY, P.B., BRIGGS, I. and DRAY, A. (1970). Antagonism of 5-hydroxytryptamine by LSD 25 in the central nervous system: a possible neuronal basis of actions of LSD 25. *British Journal of Pharmacology* **40**:202-218.

BOGDANSKI, D., WEISBACH, H. and UNDENFREIND, S. (1958). Pharmacological studies with serotonin precursor, 5-hydroxytryptophan. *Journal of Pharmacology and Experimental Therapeutics* **122**:182-194.

BOLME, P. and FUXE, K. (1973). Pharmacological studies on a possible role of central noradrenaline neurons in respiratory control. *Journal of Pharmacy and Pharmacology* **25**:351-352.

BONORA, M., ST. JOHN, W.M. and BLEDSOE, T.A. (1985). Differential elevation by protryptaline and depression by diazepam of upper airway respiratory motor activity. *American Review of respiratory Diseases* **131**:41-45.

BOWMAN, W.C. and RAND, M.J. (1980). In Textbook of Pharmacology, second edition, pp:11.1-11.49. Peripheral Adrenergic Mechanism. Blackwell Scientific Publication, London.

BRAMWELL, G.J. and BRADLEY, P.B. (1974). Actions and interactions of narcotic agonists and antagonists on brainstem neurons. *Brain Research* **73**:167-170.

BROCKHAUS, J., BALLANYI, K., SMITH, J.L. and RICHTER, D.W. (1993). Microenvironment of respiratory neurons in the *in vitro* brainstem-spinal cord neonatal rats. *Journal of Physiology (London)* **462**:421-445.

CHAMPAGNAT, J., DENAVIT-SAUBIE, M., HENRY, J.L. and LEVIEL, V. (1979). Catecholamine depressant effect on bulbar respiratory mechanisms. *Brain Research* **160**:57-68.

CHANG, F.C.T. (1993). State dependant variations in medullary respiratory related discharge patterns. Respiratory control: central and peripheral mechanisms. Eds. SPECK, D.F., DEKIN,M.S., REVELETTE, W.R. and FRAZIER D.T. The University of Kentucky press, **pp**:199-206.

CLARK, R.W., SCHMIDT, H.S., SHAAL, S.F., BOUDOULAS, H. and SHULLER, D.E. (1979). Sleep apnea: Treatment with Protriptyline. *Neurology* **29**:1287:1292.

CLEMENT, M.E and McCALL, R.B. (1991). Pharmacological characterization of medullary serotonin neurons. *Brain Research* **542**:205-211.

CONNELLY, C.A., HERNANDEZ, J.D., FINLON, E.G., OTTO-SMITH, M.R. and FELDMAN, J.L. (1991). Alteration of breathing by non-NMDA receptor blockade in pre-Bötzinger region of adult cats and rats. *Society for Neuroscience Abstracts* **17**:620.

CONNELLY, C.A., DOBBINS, E.G. and FELDMAN, J.L. (1992a). Pre-Bötzinger complex in cats: respiratory neural discharge patterns. *Brain Research* **590**:337-340.

CONNELLY, C.A., OTTO-SMITH, M.R. and FELDMAN, J.L. (1992b). Blockade of NMDA receptor-channels by MK-801 alters breathing in adult rats. *Brain Research* **596**: 99-110.

CONWAY, W.A., ZORICK, F., PICCIONE, P. and ROTH, T. (1982). Protriptyline in the treatment of sleep apnea. *Thorax* **37**:49-53.

DALSTRÖM, A. and FUXE, K. (1964). Evidence for the existence of monoamine containing neurons in the central nervous system. *Acta Physiology Scandinavia* **62** (Supplement 232):1-55.

DIPASQUALE, E., MORIN, D., MONTEAU, R. and HILAIRE, G. (1992). Serotonergic modulation of respiratory rhythm generator at birth: an *in vitro* study in rat. *Neuroscience Letters* **143**:91-95.

DOBBINS, E.G. and FELDMAN, J.L. (1994). Brainstem network controlling descending drive to phrenic motoneurons in rat. *Journal of Comparative Neurology* **347**:64-86.

DRETELER, G.H, WOUTERS, W., SAXENA, P.R. and RAMAGE, A.G. (1991) Pressor effects following microinjection of 5-HT_{1A} receptor agonists into the nucleus raphé obscurus of the anesthetized rat. *British Journal of Pharmacology* **102**:317-322.

ELDRIDGE, F.L., MILLHORN, D.E. and WALROP, T.G. (1979). Respiratory effects of centrally injected serotonin. *Physiologist* **22**:33 (*Abstract*).

ELDRIDGE, F.L. and MILLHORN, D.E. (1981). Central regulation of respiration by endogenous neurotransmitters and neuromodulators. *Annual Review of Physiology* **43**:121-135.

ELLENBERGER, H.H., VERA, P.L., FELDMAN, J.L. and HOLETS, V.R. (1992). Multiple putative neuromessenger inputs to phrenic motoneurons in rat. *Journal of Chemical Neuroanatomy* **5**:375-382.

ERRICHIDI, S., HILAIRE, G. and MONTEAU, R. (1990). Permanent release of Noradrenaline modulates respiratory frequency in the newborn rat: an *in vitro* study. *Journal of Physiology (London)* **429**:497-510.

ERRICHIDI, S., MONTEAU, R. and HILAIRE, G. (1991). Noradrenergic modulation of medullary respiratory rhythm generator in the newborn rat: an *in vitro* study. *Journal of Physiology (London)* ***43***:477-498.

FEDORKO, L., CONNELLY, C.A. and REMMERS J.E. (1987). Neurotransmitters mediating synaptic inhibition of phrenic motoneurons. Respiratory Muscle and Their Neuromotor Control. (GC Sieck, SC Gandevia and WE Cameron, Eds.). New York, Alan R. Liss, Inc. 167-173.

FELBURG, W. and SHERWOOD, S. (1954). Injections of drugs into the lateral ventricle of the cat. *Journal of Physiology (London)* **123**:148-167.

FELDMAN, J.L. (1986). Neurophysiology of breathing in mammals. Handbook of Physiology, Section 1: The Nervous System. Vol. 4, Intrinsic Regulatory Systems of the Brain. (FE BLOOM, Ed.). Bethesda, MD, American Physiology Society. 463-524.

FINDLAY, J. and THOMPSON, G. (1968). The effects of intraventricular injections of noradrenaline, 5-hydroxytryptamine, acetylcholine and trancyclpromine on the ox (*Bos taurus*) at different environmental temperatures. *Journal of Physiology (London)* **194**:809-816.

FUKUDA, A., NABEKURA, J., ITO, C., PLATA-SALAMÁMAN, C.R. and OOMURA. Y. (1989). Developmentally onset of α_1 -and α_2- adrenergic responses in the neonatal rat dorsal motor nucleus of the vagus *in vitro*. *Brain Research* **493**:357-361.

FUNK, G.D., SMITH J.C. and FELDMAN, J.L. (1993). Generation and transmission of respiratory oscillations in medullary slices: role of excitatory amino acids. *Journal of Neurophysiology* **70**:1497-1515.

FUNK, G.D., SMITH, J.C. and FELDMAN, J.L. (1994). Development of thyrotropin-releasing hormone and norepinepherine potentiation of inspiratory-related hypoglossal motoneuron discharge in neonatal and juvenile mice *in vitro*. *Journal of Neurophysiology* **72**:2538-2541.

FUXE, K. (1965). Evidence for the existence of monoamine neurons in the central nervous system. IV. Distribution of monoamine nerve terminals in the central nervous system. *Acta Physiology Scandinavia* (Supplement 247) **64**:39-85.

GADDUM, H. and VOGT, M. (1956). Some central actions 5-hydroxytryptamine and various antagonists. *British Journal of Pharmacology* **11**:175-179.

GREER, J.J., SMITH, J.C. and FELDMAN, J.L. (1991). Role of excitatory amino acids in the generation and transmission of respiratory drive in neonatal rat. *Journal of Physiology (London)* **437**:727-749.

GREER, J.J., SMITH, J.C. and FELDMAN, J.L. (1992a). Glutamate release and presynaptic action of AP4 during inspiratory drive to phrenic motoneurons. *Brain Research* **576**:355-357.

GREER, J.J., SMITH, J.C. and FELDMAN, J.L. (1992b) Respiratory and locomotor patterns generated in the fetal rat brain stem spinal-cord *in vitro*. *Journal of Neurophysiology* **67**:996-999.

GREER, J.J., AL-ZUBAIDY, Z.A. and CARTER, J. (1995a). Thyrotropin releasing hormone (TRH) stimulates respiratory rhythm generating centre in the perinatal rat. *Submitted to American Journal of Physiology.*

GREER, J.J., CARTER, J. and AL-ZUBAIDY, Z.A. (1995b). Opioid depression of breathing in neonatal rats. *Journal of Physiology (London)* **485**:845-855.

HILAIRE, G., MONTEAU, R. and ERRICHIDI, G. (1989). Possible modulation of medullary respiratory rhythm generator by the noradrenergic A5 area: an *in vitro* study in the new born rat. *Brain Research* **485**:325-332.

HÖLSI, L., TEBECIS, A.K. and SCHÖNWETTER, H.P. (1971). A comparison of the effects of monoamines on neurons of the bulbar reticular formation. *Brain Research* **25**:357-370.

HOLTMAN, J., ANASTASI, N., NORMAN, W. and DRETCHEN, K. (1986). Effect of electrical stimulation of the raphé obscurus on phrenic nerve activity in the cat. *Brain Research* **362**:214-220.

HOLTMAN, J., DICK, T. and BERGER, A. (1987). Serotonin-mediated excitation of recurrent laryngeal and phrenic motoneurons evoked by stimulation of the raphé obscurus. *Brain Research* **417**:12-20.

HOLTMAN, J. (1990). Immunohistochemical localization of serotonin- and substance P-containing fibres around respiratory muscle motoneurons in the nucleus ambiguus of the cat. *Neuroscience* **26** (1):169-179.

HOLTMAN, J., KING, K. (1994). Effect of activation of 5-HT_{1A} receptor at the ventral medulla on phrenic nerve activity. *European Journal of Pharmacology* **253**:307-310.

JOUVET, M. (1969). Biogenic amines and the state of sleep. *Science* **16**:32-41.

JONES, B.E. (1991) Paradoxical sleep and its chemical/structural substrates in the brain. *Neuroscience* **40**:637-656.

KUBIN,L., TOJIMA, H., DAVIES, R.O. and PACK, A.I. (1992). Serotonergic excitatory drive to hypoglossal motoneurons in the decerebrate cat. *Neuroscience Letters* **139**: 243-248.

KUBIN, L., REIGNER, C., TOJIMA, H., TAGUCHI, O., PACK, A.I. and DAVIES, R.O. (1994). Changes in serotonin level in the hypoglossal nucleus region during carbachol-induced atonia. *Brain Research* **645**:291-302.

LALLEY, P.M. (1986). Responses of phrenic motoneurons of cat to stimulation of medullary raphé nuclei. *Journal of Physiology (London)* **380**:349-371.

LALLEY, P.M., BISCHOFF, A.M. and RICHTER, D.W. (1994). 5-HT_{1A} receptor-mediated modulation of medullary expiratory neurones in the cat. *Journal of Physiology (London)* **476**:117-130.

LAMBERT, G., FRIEDMAN, E., BUSCHWERTZ, E. and GERSHON, S. (1978). Involvement of 5-hydroxytryptamine in the central control of respiration, blood pressure and heart rate in anesthetized rat. *Neuropharmacology* **17**:807-813.

LEVITT, P. and MOORE, R.Y. (1979). Origin and organization of brainstem catecholamine innervation in the rat. *Journal of Comparative Neurology* **186**:505-528.

LINDSAY, A.D. and FELDMAN, J.L. (1993). Modulation of respiratory activity of neonatal rat phrenic motoneurons by serotonin. *Journal of Physiology (London)* **461**:213:233.

LUI, G., FELDMAN, J.L. and SMITH, J.C. (1990). Excitatory amino acid mediated transmission of inspiratory drive to phrenic motoneurons. *Journal of Neurophysiology* **64**: 423-436.

McCRIMMON, D. and LALLEY, P. (1982). Inhibition of respiratory neural discharges by clonidine and 5-hydroxytryptophan. *Journal of Pharmacology and. Experimental Therapeutics* **222**:771-777.

McCRIMMON, D.R., SMITH, J.C. and FELDMAN, J.L. (1989). Involvement of excitatory amino acids in neurotransmission of inspiratory drive to spinal respiratory motoneurons. *Journal of Neuroscience* **9**:1910-1921.

MILLHORN, D., ELDRIDGE, F. and WALROP, T. (1980). Prolonged stimulation of respiration by a new central neural mechanism. *Respiratory Physiology* **41**:87-103.

MILLHORN, D.E. (1986). Stimulation of raphé (obscurus) nucleus causes long-tern potentiation of phrenic nerve activity in cats. *Journal of Physiology (London)* **381**:169-179.

MONTEAU, R., MORIN, D., HENNIQUIN, S.A. and HILAIRE, G. (1990). Differential effects of serotonin on respiratory activity of hypoglossal and cervical motoneuron: an *in vitro* study on the newborn rat. *Neuroscience Letters* **111**:127-132.

MORIN, D., HENNEQUIN, S., MONTEAU, R. and HILAIRE, G. (1990a). Serotonergic influences on central respiratory activity: an *in vitro* study in the newborn rat. *Brain Research* **535**:281-287.

MORIN, D., HENNEQUIN, S., MONTEAU, R. and HILAIRE, G. (1990b). Depressant effect of raphé stimulation on inspiratory activity of the hypoglossal nerve: an *in vitro* study in the newborn rat. *Neuroscience Letters* **116**:299-303.

MORIN, D. (1993). Compared effects of serotonin on inspiratory activity of glossopharyngeal, vagal, hypoglossal and cervical motoneurons in neonatal rat brainstem spinal-cord preparation. *Neuroscience Letters* **160**:61-64.

MOSS, I.R. and INMAN, J.G. (1989). Neurochemicals and respiratory control during development. *Journal of Applied Physiology* **67**:1-13.

MULLER, R., LUNDBERG, D. and BREESE, G. (1980). Evidence that respiratory depression by serotonin agonists may be exerted in the central nervous system. *Pharmacology, Biochemistry and Behaviour* **13** (2):247-258.

MURAKOSHI, T., SUZUE, T. and TAMAI, S. (1985). A pharmacological study on respiratory rhythm in the isolated brainstem-spinal cord preparation of the newborn rat. *British Journal of Pharmacology* **86**:95-104.

OLSON, E., DEMPSEY, J. and McCRIMMON, D. (1979) Serotonin and control of ventilation in awake rats. *Journal of Clinical Investigations* **64**:689-693.

ONIMARU, H., HOMMA, I and IWATSUKI, K. (1992). Excitation of inspiratory neurons by preinspiratory neurons in rat medulla *in vitro*. *Brain Research Bulletin* **29**:879-882.

OREM, J. (1994). Central respiratory activity in rapid eye movement sleep: Augmenting and late inspiratory cells. *Sleep* **17**:665-673.

PARKIS, M.A., BAYLIS, D.A. and BERGER, A.J. (1994). At least two distinct ionic mechanisms underlie the response of hypoglossal motoneurons (HMs) to norepinepherine. *Society for Neuroscience Abstracts* **20**:1589.

PEROUTKA,S.J., SCHMIDT, A.W., SLEIGHT, A.J. and HARRINGTON, M.A. (1990). Serotonin Receptor "Families" in the Central Nervous System: An overview. *Annals New York Academy of Sciences* **600**:104-113.

PILOWSKI, P.M., JIANG C. and LIPSKI J. (1990). An intracellular study of respiratory neurons in the rostral ventrolateral medulla of the rat and their relationship to catecholamine-containing neurons. *Journal of Comparative Neurology* **301**:604-617.

PILOWSKI, P., LLEWELLYN-SMITH, I.J., LIPSKI, J., MINSON, J., ARNOLDA, L. and CHARMERS, J. (1994). Projections from inspiratory neurons of the ventral respiratory group to the subretrofacial nucleus of the cat. *Brain Research* **633**:63-71.

RICHTER, D.W. (1982). Generation and maintenance of respiratory rhythm. *Journal of Experimental Biology* **100**:93-107.

RODRIGUEZ, A.M., WARBURTON, D. and KEENS, T.G. (1987). Elevated catecholamine levels and abnormal hypoxic arousal in apnea of infants. *Pediatrics* **79**:269-274.

SCHER, M.J., GUTHRIE, R.D., KRIEGER, D., SUN, M. and SCALABASSI, R. (1992). Maturational aspects of sleep from birth through early childhood. Respiratory Control Disorders in Infants and Children. Williams and Wilkins, Baltimore, **pp**: 352-370.

SCHWARZACHER, S., SMITH, J.C. and RICHTER, D.W. (1995). Pre-Bötzinger complex in the cat. *Journal of Neurophysiology* **73 (4)**:1452-1461.

SLOVITER, R., DRUST, E. and CONNOR, J. (1978). Serotonin agonist actions of p-chlorophenylalanine. *Neuropharma*cology **17**:1029-1033.

SMITH, J.C., GREER, J.J., LUI, G. and FELDMAN, J.L. (1990). Neural mechanisms generating respiratory pattern in mammalian brainstem-spinal cord *in vitro*. I. Spatiotemporal patterns of motor and medullary neuron activity. *Journal of Neurophysiology* **354**:173-183.

SMITH, J.C., ELLENBERGER, H.H., BALLANYI, K., RICHTER, D.W. and FELDMAN, J.L. (1991). Pre-Bötzinger complex: A brainstem region that may generate respiratory rhythm in mammals. *Science* **254:**726-728.

SMITH, J.C., FUNK, G.D., JOHNSON, S.M. and FELDMAN, J.L. (1992). A hybrid pacemaker-network model for the respiratory oscillator in mammals. *Society of Neuroscience Abstract* **18**:1279.

SUN, Q.,PILOWSKI, P., MINSON, J., ARNOLA, L., CHARMERS, J. and LLEWELLYN-SMITH, I.J. (1994). Close appositions between tyrosine hydroxylase immunoreactive boutons and respiratory neurons in the rat ventrolateral medulla. *Journal of Comparative Neurology* **340**:1-10

SUZUE, T. (1984). Respiratory rhythm generation in the *in vitro* brainstem and spinal cord preparation of the newborn rat. *Journal of Physiology (London)* **354**:173-183.

TÖRK, I. (1985). Raphé nuclei and serotonin containing systems. In *Ed. Paxinos, G. The Rat Nervous System, Vol. 2, Hindbrain and spinal cord.* Academic Press, Orlando, pp.43-78.

WILLIAMS, J.A. and REINER, P.B. (1993). Noradrenaline hyperpolarizes identified rat mesopontine cholinergic neurons *in vitro*. *Journal of Neuroscience* **13**:3878-3993.

YAMAMOTO, Y., ONIMARU, H. and HOMMA, I. (1992). Effect of substance P on respiratory rhythm and pre-inspiratory neurons in the ventrolateral structure of rostral medulla oblongata: an *in vitro* study. *Brain Research* **599**:272-276.

ZOU, D.J. (1994). Respiratory rhythm in the isolated nervous system of the newborn opossum. *Journal of Experimental Biology* **197**:201-213.